VIRAL VECTORS ENCODING APOPTOSIS-INDUCING PROTEINS AND METHODS FOR MAKING AND USING THE SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119(e) from U.S. Provisional Application Serial No. 60/134,416, filed May 17, 1999, entitled "Product for Production of Apoptosis-Inducing Proteins and Uses Therefor". This application is also a continuation-in-part of copending U.S. Patent Application Serial No. 09/087,195, filed May 29, 1998, entitled "Use of Fas Ligand to Suppress T-Lymphocyte-Mediated Immune Response", which is a continuation of U.S. Patent Application Serial No. 08/378,507, now U.S. Patent No. 5,759,536, issued June 2, 1998, entitled "Use of Fas Ligand to Suppress T-Lymphocyte-Mediated Immune Response", which is a continuation-in-part of U.S. Patent Application Serial No. 08/250,478, filed May 27, 1994, entitled "Use of Fas Ligand to Suppress T-Lymphocyte-Mediated Immune Response", now abandoned. The entire disclosures of U.S. Provisional Application Serial No. 60/134,416, U.S. Patent Application Serial No. 09/087,195, U.S. Patent No. 5,759,536 and U.S. Patent Application Serial No. 08/250,478 are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention generally relates to a method for propagating viral vectors encoding proteins that induce apoptosis, and to products and methods related to the used of such viral vectors. In particular, the present invention relates to methods of using viral vectors of the present invention in methods for suppressing T-lymphocyte-mediated graft rejection and T-lymphocyte-mediated disease and for inducing apoptosis in cancer cells.

BACKGROUND OF THE INVENTION

Apoptosis is a regulated form of cell death that is necessary for normal cell function and development. The regulation of apoptosis in a cell is mediated by diverse signals and complex interactions of many different cellular gene products. Dysregulation of apoptosis can cause or contribute to a variety of diseases and conditions. However, having the ability

to induce apoptosis in target cells in certain disease states would be extremely advantageous. Over the past several years, numerous gene products which modulate the apoptotic process have been identified. The discovery of such gene products presents opportunities to intervene in normal and abnormal cellular processes and regulate cell death for therapeutic purposes.

Fas is one such gene product. Fas (CD95/APO-1) is a transmembrane glycoprotein that is related to the receptors for tumor necrosis factor and nerve growth factor (Itoh et al., 1991, *Cell* 66:23-243; Oehm et al., 1992, *J. Biol. Chem.* 267:10709-10715). Upon being cross-linked with agonistic anti-Fas antibodies or Fas ligand (FasL), Fas initiates a complex signal transduction pathway that, in sensitive cell types, ultimately ends in apoptotic cell death. The Fas/FasL pathway is probably best recognized for its role in the downregulation of expanded clonal T lymphocyte populations. In this system, Fas is upregulated within a few hours of T cell activation (Miyawaki et al., 1992, *J. Immunol.* 149:3753-3758). Several days later, Fas becomes functional and if the cells continue to be stimulated through the antigen receptor, FasL is also upregulated (Owen-Schaub et al., 1992, *Cell. Immunol.* 140:197-205; Suda et al., 1993, *Cell* 75:1169-1178), and the majority of activated cells undergo apoptosis, allowing the immune system to return to its normal resting size and repertoire.

In related U.S. Patent No. 5,759,536, the present inventors disclosed that a factor released by testicular Sertoli cells, which is responsible for the protection of the intratesticular islet allografts and xenografts against rejection, is the Fas ligand. U.S. Patent No. 5,759,536 disclosed the use of Fas ligand to suppress graft rejection, to suppress T lymphocyte-mediated disease, and to suppress T lymphocyte-mediated disease recurrence. Fas ligand mediates its effect by interacting with Fas. As discussed in detail in U.S. Patent No. 5,759,536, a major problem associated with transplantation of any tissue is immune-mediated graft rejection in which the recipient's T-lymphocytes recognize donor histocompatibility antigens as foreign. Current regimes for transplanting many tissues and organs require lifelong administration of immunosuppressive drugs. These drugs have serious side-effects and can cause increased susceptibility to infection, renal failure,

hypertension, and tumor development. Fas ligand/Fas-mediated apoptosis provided a novel solution to these problems.

In addition to regulating immune responses, Fas and its ligand are likely to play a role in other systems as well. For example, the testes and placenta, both of which are known to be immune-privileged tissues, express FasL (Xerri et al., 1997, *Mol. Pathol.* 50:87-91). Additionally, Fas and FasL have been found to be coexpressed in a few epithelial tissues that are marked by apoptotic cell turnover, such as the uterus and prostate (Leithauser et al., 1993, *Lab. Invest.* 69:415-429; French et al., 1996, *J. Cell. Biol.* 133:335-343; and Xerri et al., 1997, *supra*). Both of these tissues are steroid-dependent and undergo apoptosis within 24-48 h after hormone depletion (Issacs et al., 1992, *J. Androl.* 19:457-464; Rotello et al., 1992, *Am. J. Pathol.* 140:449-456). Interestingly, the apoptosis that occurs in response to steroid depletion has recently been shown to require sufficient Fas expression (Suzuki et al., 1996, *EMBO. J.* 15:211-215; Suzuki et al., 1996, *Oncogene* 13:31-37). These data support a role for the Fas signaling pathway in the normal renewal of the uterine and prostatic epithelium.

In addition to the *in vivo* data above, several laboratories have recently demonstrated a potential role for Fas-dependent apoptosis in human prostate cancer (PC) cell lines (Rokhlin et al., 1997, *Cancer Res.* 57:1756-1758; Uslu et al., 1997, *Clin. Cancer Res.* 3:963-972; Hedlund et al., 1998, *Prostate* 36:92-101). Although Fas expression has proved to be a common feature of the cell lines studied, contradictory results were reported with regard to their apoptotic potentials. This may be due to the use of different agonistic anti-Fas antibodies among laboratories, or to the different experimental conditions that were employed. Taken as a whole, these studies indicated that the apoptotic potential of cells expressing Fas may not be sufficient to enable the widespread use of Fas ligand as a therapeutic agent.

In addition, the ability to produce Fas ligand in a form and quantity which is readily useable for both *in vitro* and *in vivo* scientific and clinical protocols has been a problem experienced by several researchers. In particular, production of viral vectors encoding apoptosis-inducing proteins such as Fas ligand has met with limited success, due to massive death of the cells used to package and/or deliver such vector and/or unsuitably low viral

production (Larregina et al., 1998, *Gene Therapy* 5:563-568; Muruve et al., 1997, *Hum. Gene. Ther.* 8:955-963; Arai et al., 1997, *PNAS USA* 94:13862-13867; Kang et al., 1997, *Nature Med.* 3:738-743). Such problems are particularly apparent when efforts have been made to scale up production of the vector. This problem of autocrine regulation can be generally extended to other apoptosis-inducing proteins. Therefore, although Fas ligand and/or other apoptosis-inducing proteins appear to be ideal candidates for various therapeutic protocols, including suppression of graft rejection, suppression of T-lymphocyte-mediated disease, and treatment of cancers, unexpected problems with the production and use of such proteins have hindered efforts to design useful therapeutic strategies using such agents.

Therefore, there is a need in the art for a safe and effective agent, such as a construct encoding Fas ligand and/or other apoptosis-inducing proteins, which is capable of inducing apoptosis in a desired target cell.

SUMMARY OF THE INVENTION

One embodiment of the present invention relates to a method to propagate a recombinant viral vector comprising a nucleic acid sequence encoding an apoptosis-inducing protein. The method includes the step of culturing an isolated cell transfected with: (a) a recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a protein that inhibits apoptosis operatively linked to a transcription control sequence; and, (b) a recombinant viral vector comprising a nucleic acid sequence encoding a protein that induces apoptosis operatively linked to a transcription control sequence. The isolated cell is cultured under conditions effective to propagate the recombinant viral vector. In one embodiment, the method additionally includes the step of recovering the recombinant viral vector from the isolated cell. Preferably, the isolated cell is a mammalian cell.

In one embodiment, the recombinant nucleic acid molecule of (a) is contained within the recombinant viral vector of (b). In this aspect, the nucleic acid sequence of (a) and the nucleic acid sequence of (b) can be operatively linked to different transcription control sequences or to the same transcription control sequences. In one aspect, the nucleic acid

sequence of (a) and the nucleic acid sequence of (b) are separated by an internal ribosome entry site (IRES).

The protein that inhibits apoptosis can include inhibitors of caspase-8 family activation and inhibitors of caspase-9 family activation. In one embodiment, the protein that inhibits apoptosis is a protein having biological activity of a protein which includes, but is not limited to, cowpox virus caspase inhibitor (CrmA), baculovirus p35, inhibitor of apoptosis protein (IAP), dominant negative Fas-associating death domain-containing protein (dominant negative FADD), dominant negative Fas, FADD-like ICE inhibitory protein (FLIP), Bcl-2, Bcl-X_L, and adenovirus E1B-19K protein. Preferred nucleic acid sequences encoding a protein that inhibits apoptosis encode a protein comprising an amino acid sequence selected from the group of SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, and positions 80-208 of SEQ ID NO:14. Preferred nucleic acid sequences encoding a protein that inhibits apoptosis include SEQ ID NO:37, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, and positions 367-753 of SEQ ID NO:13. In a preferred embodiment, the protein that inhibits apoptosis is a protein having CrmA biological activity.

The protein that induces apoptosis can include, but is not limited to a protein that has biological activity of a protein selected from the group consisting of Fas ligand, Fas, Fas-associating death domain-containing protein (FADD), Fas-associated death domain-like IL-1β converting enzyme (FLICE), tumor necrosis factor (TNF), TWEAK/Apo3L, TRAIL/Apo2L, Bax, Bid, Bik, Bad, Bak, and RICK. Preferred proteins that induces apoptosis comprise an amino acid sequence selected from the group of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34 and SEQ ID NO:36. Preferred nucleic acid sequences encoding a protein that induces apoptosis is selected from the group consisting of SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33 and SEQ ID NO:35.

In one embodiment, the recombinant viral vector is packaging deficient. In another embodiment, the recombinant viral vector is replication deficient. The recombinant viral vector is preferably from a virus including, but not limited to, alphaviruses, poxviruses, adenoviruses, herpesviruses, lentiviruses, adeno-associated viruses, vaccinia viruses, baculoviruses, parvoviruses and retroviruses. In a preferred embodiment, the recombinant viral vector is from an adenovirus. In one aspect, the recombinant viral vector comprises a human adenovirus 5 construct under the control of a CMV immediate early promoter. Such a human adenovirus 5 construct can be replication deficient. In another embodiment, the recombinant viral vector comprises a nucleic acid sequence represented by at least a portion of SEQ ID NO:4.

Preferably, the present method results in the isolated cell producing at least about 1 x 10^8 plaque forming units (pfu) of the recombinant viral vector per ml of supernatant isolated from the cell, and more preferably, at least about 5 x 10^8 pfu, and more preferably, at least about 1 x 10^9 pfu of the recombinant viral vector per ml of supernatant isolated from the cell.

Another embodiment of the present invention relates to an isolated cell, wherein the cell is transfected with: (a) a recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a protein that inhibits apoptosis operatively linked to a transcription control sequence; and, (b) a recombinant viral vector comprising a nucleic acid sequence encoding a protein that induces apoptosis operatively linked to a transcription control sequence. In one embodiment, the recombinant nucleic acid molecule of (a) is contained within the recombinant viral vector of (b). In this aspect, the nucleic acid sequence of (a) and the nucleic acid sequence of (b) can be operatively linked to different transcription control sequences. Alternatively, the nucleic acid sequence of (a) and the nucleic acid sequence of (b) can be separated by an internal ribosome entry site (IRES). Other preferred embodiments of such a recombinant viral vector and recombinant molecule are as disclosed above.

Another embodiment of the present invention relates to a recombinant viral vector for inducing apoptosis in cells transfected with the vector. The viral vector comprises a

recombinant virus comprising: (a) an isolated nucleic acid sequence encoding a protein that inhibits apoptosis operatively linked to a transcription control sequence; and, (b) an isolated nucleic acid sequence encoding a protein that induces apoptosis operatively linked to a transcription control sequence. In one embodiment, the nucleic acid sequence of (a) and the nucleic acid sequence of (b) are operatively linked to different transcription control sequences. In another embodiment, the nucleic acid sequence of (a) and the nucleic acid sequence of (b) are separated by an internal ribosome entry site (IRES). Other preferred embodiments of such a recombinant viral vector and recombinant molecule are as disclosed above. In one embodiment, the recombinant viral vector comprises a nucleic acid sequence represented by at least a portion of SEQ ID NO:4.

Another aspect of the present invention relates to a recombinant viral vector comprising: (a) an isolated human adenovirus 5 construct encoded by a nucleic acid sequence comprising at least a portion of SEQ ID NO:4; and, (b) a recombinant nucleic acid molecule comprising a nucleic acid sequence encoding Fas ligand or a biologically active fragment thereof, operatively linked to a transcription control sequence. More particular aspects of the viral vector and recombinant nucleic acid molecule have been disclosed above.

Yet another embodiment of the present invention relates to a method of suppressing T-lymphocyte-mediated graft rejection in a recipient mammal, the method comprising introducing into the mammal a pharmaceutically acceptable carrier comprising a recombinant viral vector comprising a nucleic acid sequence encoding a protein that induces apoptosis operatively linked to a transcription control sequence, wherein the recombinant viral vector expresses the protein that induces apoptosis. The pharmaceutically acceptable carrier can further comprise a recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a protein that inhibits apoptosis operatively linked to a transcription control sequence. In this aspect, the recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a protein that inhibits apoptosis can be contained within the recombinant viral vector. In this aspect, the nucleic acid sequence encoding a protein that inhibits apoptosis and the nucleic acid sequence encoding a protein that induces apoptosis can be operatively linked to different transcription control sequences or alternatively, the nucleic

acid sequence encoding a protein that inhibits apoptosis and the nucleic acid sequence encoding a protein that induces apoptosis can be separated by an internal ribosome entry site (IRES).

In one embodiment, the pharmaceutically acceptable carrier is an isolated cell that is transfected with the recombinant nucleic acid molecule and the recombinant viral vector and/or a pharmaceutically acceptable excipient. In one aspect, the isolated cell is a cell of the graft. In another embodiment, the cell is not a part of the graft. In another embodiment, the cell is an islet cell.

Another embodiment of the present invention relates to a method of inducing apoptosis in cancer cells of a recipient mammal, comprising introducing into the mammal a recombinant viral vector comprising: (a) a recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a protein that inhibits apoptosis operatively linked to a transcription control sequence; and, (b) a recombinant viral vector comprising a nucleic acid sequence encoding a protein that induces apoptosis operatively linked to a transcription control sequence. The protein that induces apoptosis is expressed by a cell at or adjacent to a site of the cancer, and the expression of the protein at the site of the cancer is sufficient to produce a result selected from the group of: reduction of tumor size, elimination of tumor cells at the site; prevention of tumor growth at the site and prevention of metastases from the tumor cells. The cancer can include, but is not limited to, lung cancer, brain cancer, prostate cancer, lymphoma and leukemia.

Yet another embodiment of the present invention relates to a method of suppressing a T-lymphocyte-mediated disease in a recipient mammal, the method comprising introducing into the mammal a pharmaceutically acceptable carrier comprising a recombinant viral vector comprising a nucleic acid sequence encoding a protein that induces apoptosis operatively linked to a transcription control sequence, wherein the recombinant viral vector expresses the protein that induces apoptosis. In this embodiment, the pharmaceutically acceptable carrier can further comprise a recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a protein that inhibits apoptosis operatively linked to a transcription control sequence. In this aspect, the recombinant nucleic acid molecule comprising a nucleic

acid sequence encoding a protein that inhibits apoptosis can be contained within the recombinant viral vector. The nucleic acid sequence encoding a protein that inhibits apoptosis and the nucleic acid sequence encoding a protein that induces apoptosis can be operatively linked to different transcription control sequences or alternatively, the nucleic acid sequence encoding a protein that inhibits apoptosis and the nucleic acid sequence encoding a protein that induces apoptosis can be separated by an internal ribosome entry site (IRES). The pharmaceutically acceptable carrier can be an isolated cell that is transfected with the recombinant nucleic acid molecule and the recombinant viral vector and/or a pharmaceutically acceptable excipient. In this embodiment of the invention, the T lymphocyte-mediated disease can include, but is not limited to, rheumatoid arthritis, insulin-dependent diabetes mellitus, multiple sclerosis, lupus erythematosus, myasthenia gravis, and graft versus host reactions.

BRIEF DESCRIPTION OF THE DRAWINGS OF THE INVENTION

Fig. 1A is a bar graph illustrating that K562-hFasL induces lysis of L1210-Fas in a dose-dependent fashion.

Fig. 1B is a bar graph illustrating that K562-hFasL induces lysis of PC cell line ALVA-31 in a dose-dependent fashion.

Fig. 2A is a graph showing Fas expression by 293 cells.

Fig. 2B is a bar graph showing that 293 cells are extraordinarily sensitive to the lytic effects of K562-hFasL.

Fig. 2C is a bar graph showing that 293-crmA transfectants are almost entirely resistant to K562-hFasL.

Fig. 3 is a line graph illustrating that ALVA-31 cells are far more sensitive to the natural FasL protein than they are to agonistic anti-Fas antibody.

Fig. 4A is a line graph showing the effect of Ad-FasL transduction on short-term growth curves of prostate cancer cell line PPC-1.

Fig. 4B is a line graph showing the effect of Ad-FasL transduction on short-term growth curves of prostate cancer cell line JCA-1.

Fig. 4C is a line graph showing the effect of Ad-FasL transduction on short-term growth curves of prostate cancer cell line PC-3.

Fig. 4D is a line graph showing the effect of Ad-FasL transduction on short-term growth curves of prostate cancer cell line TSU-Pr1.

Fig. 5 is a schematic drawing showing the interactions of various apoptosis-inducing proteins and apoptosis-inhibiting proteins.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is generally related to the present inventors' discovery of a novel system for the propagation of viral vectors wherein autocrine regulation by production of the protein is problematic. Specifically, the present invention is related to the discovery that it is possible to successfully produce and propagate viral vectors encoding apoptosis-inducing proteins in significant quantities without destroying the production cell line or experiencing problems with production levels that have been observed prior to the present invention. The present invention relates to the use of a combination of a novel viral delivery vector encoding an apoptosis-inducing protein and an isolated nucleic acid molecule encoding an apoptosis-inhibiting protein to propagate the viral vector encoding the apoptosis-inducing protein. In addition, the present invention relates to the use of the viral vector encoding an apoptosis-inducing protein, alone or combined with an isolated nucleic acid molecule encoding an apoptosis-inhibiting protein, in a product and method to suppress graft rejection, to suppress T-lymphocyte mediated diseases, and to treat cancers.

Several laboratories have reported on the apoptotic potentials of human prostate cancer (PC) cell lines in response to crosslinking of Fas (CD95/APO-1) with agonistic anti-Fas antibodies. As discussed above, however, contradictory results have been reported with regard to their apoptotic potentials. Moreover, soluble FasL, while once appearing to be a desirable means of using Fas ligand as a therapeutic tool, is now known to sometimes be ineffective for the induction of apoptosis in a Fas-bearing cell in at least some scenarios (Tanaka et al., 1998, *J. Exp. Med.* 187:1205-1213). In addition, while researchers have focused considerable effort on the production of viral vectors encoding Fas ligand and other

apoptosis-inducing proteins for use in suppression of graft rejection, tumor reduction, or suppression of T-lymphocyte-mediated disease, problems with massive cell death of the packaging or delivery cell lines and/or inadequate viral titers have shed doubt on the ability of such vectors to realistically be used in therapeutic methods. Some researchers have proposed potential solutions to the problem of producing viral vectors expressing Fas ligand. For example, Arai et al., 1997, *supra* describe the use of soluble caspase inhibitor, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone, in the cell culture medium, and/or use of a Fas-ligand resistant clone of 293 cells to produce Fas ligand-viral vectors. Kanagae et al. suggest cotransfecting production cell lines with a second vector which regulates the expression of the gene of interest by inducing or inhibiting expression on demand (Kanagae et al, 1995, *Nuc. Acids Res.* 23:3816-3821). Yoshida et al. have suggested the use of an inducible promoter to produce other problematic cytotoxic products (VSVG protein) by a viral vector (Yoshida et al., 1995, *Biochem. Biophys. Res. Commun.* 232:379-382). These methods, however, are either inadequate, more time consuming and difficult, or are less effective than the method discovered by the present inventors and described herein. Moreover, the method and constructs of the present invention result in the production of a vector which can be used *in vivo* without additional manipulations of the subject which would be required by the other methods.

The present inventors have developed a viral vector system which employs cells transfected with both a viral vector encoding an apoptosis-inducing protein and an isolated nucleic acid molecule encoding an apoptosis-inhibiting protein, to enable the propagation of viral vectors encoding such apoptosis-inducing proteins, as well as the subsequent use of the vectors to express the proteins wherein such propagation and production is typically problematic. By way of example, the present inventors have developed a novel adenoviral expression system for FasL by a viral construct and have employed 293 cells that stably express CrmA, a Cowpox virus inhibitor of apoptosis, to propagate the viral construct. The present inventors have further determined the effects of FasL when expressed internally by cell lines. The vectors described herein can be used in a variety of methods as described

generally for the apoptosis-inducing protein, Fas ligand, in U.S. Patent No. 5,759,536, which is incorporated herein by reference in its entirety.

The present inventors have also re-evaluated the apoptotic potentials of seven human prostate cancer (PC) cell lines using the natural Fas ligand (FasL) in place of agonistic antibody. First, PC cell lines were tested in a standard cytotoxicity assay with a transfected cell line that stably expresses human FasL. The present inventors' data demonstrate that viral vectors encoding Fas ligand can be efficiently and effectively produced in significantly high titers in multiple cell types without destroying the producing cell line. In addition, the present inventors have discovered that the apoptotic potentials of PC cell lines have been greatly underestimated in previous studies utilizing agonistic anti-Fas antibodies. The present inventors' data further demonstrate that internally expressed Fas ligand is more effective than exposure of cellular Fas to an external source of Fas ligand. Lastly, adenoviral-mediated expression of FasL prevented growth and induced regression of two human PC cell lines in immunodeficient mice. These *in vivo* results illustrate a use for adenovirus encoding apoptosis-inducing proteins such as FasL as a gene therapy for diseases such as PC, and illustrate another means of administering FasL to a patient to suppress graft rejection and/or T-lymphocyte mediated disease as disclosed in U.S. Patent No. 5,759,536.

During the course of the present inventors' research, Arai and colleagues addressed similar questions regarding the effects of FasL expression on the growth of colon cancer cell lines in mice (Arai et al., 1997, *ibid.*). Overall, their findings strongly support the therapeutic potential of FasL transgene expression in causing the regression of tumors. Furthermore, their data suggest that this favorable response is not only observed in cell lines that undergo apoptosis in response to FasL expression *in vitro*. In fact, tumor regression also occurs in a Fas-negative (and FasL-insensitive) cell line, and regression is associated with a marked infiltration by neutrophils, many of which appear apoptotic. However, Arai and colleagues did not address the issue of how to produce a viral vector encoding Fas ligand in a manner that results in propagation of a vector in sufficient quantities and in a form that is readily administered to a patient for the treatment of cancer (e.g., in the absence of other manipulations of the patient and/or vector). Subsequent to the present invention, Shinoura

and colleagues confirmed the present inventors' results for production of viral vector encoded Fas ligand and Fas by describing another adenoviral vector-Fas ligand construct and an adenoviral vector-Fas construct, both of which were successfully constitutively produced at high titers by using CrmA-transfected 293 cells (Shinoura et al., 1998, *Human Gene Therapy* 9:2683-2689). The results of Shinoura et al. confirm the present inventors' discovery that a biological system using CrmA-transfected cells is useful for producing viral vectors encoding apoptosis-inducing proteins.

The present inventors' discovery of the novel biological system for the propagation of viral vectors encoding apoptosis-inducing proteins is extremely valuable for its application to the suppression of graft rejection, the suppression of T-lymphocyte mediated diseases, and the treatment of cancers, since now the production of such viral vectors in high titers is possible.

Reference will now be made in detail to useful embodiments of the invention, which, together with the following examples and claims, serve to explain the principles of the invention. It is to be understood that this invention is not limited to the specific examples described, and as such may, of course, vary. it is also to be understood that the terminology used herein is with the purpose of describing particular embodiments only, and is not intended to limit the scope of the invention which will be limited only by the amended claims. In addition, discussion of various embodiments of the invention is made with particular reference to Fas ligand, although it is to be understood that Fas ligand serves as an exemplary apoptosis-inducing protein, and that other apoptosis-inducing proteins are encompassed by the present invention and the discussion relates also to such proteins.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. Although any methods and materials similar to or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to describe and disclose specific information with which the reference was cited in connection.

One embodiment of the present invention relates to a method to propagate a recombinant viral vector comprising a nucleic acid sequence encoding an apoptosis-inducing protein, to the recombinant viral vectors produced by such a method and to isolated cells transfected with such recombinant viral vectors. Specifically, this method of the present invention includes the step of culturing an isolated cell (also referred to herein as a production cell line) that has been cotransfected with: (a) a recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a protein that inhibits apoptosis operatively linked to a transcription control sequence; and, (b) a recombinant viral vector comprising a nucleic acid sequence encoding a protein that induces apoptosis operatively linked to a transcription control sequence. The step of culturing is performed under conditions that are effective to propagate the recombinant viral vector (discussed in detail below). The method can additionally include the step of recovering the recombinant viral vector from the isolated cell, although in certain aspects of the present invention, it is desirable to use the transfected isolated cell in a therapeutic composition or method, as discussed below. Cell lines and/or other pharmaceutically acceptable carriers which contain the recombinant viral vector comprising a nucleic acid sequence encoding an apoptosis-inducing protein, alone or in combination with a recombinant nucleic acid molecule comprising a nucleic acid sequence encoding an apoptosis-inhibiting protein, can be used in a therapeutic composition for suppressing T-lymphocyte-mediated graft rejection, for suppressing T-lymphocyte-mediated disease in a recipient mammal, and/or for inducing apoptosis in cells of a mammal, such as in cancer cells. These embodiments are discussed in detail below.

According to the present invention, the term "apoptosis" is defined as a regulated form of cell death that is necessary for normal cell function and development. As used herein, apoptosis refers to a form of cell death that includes progressive contraction of cell volume with the preservation of the integrity of cytoplasmic organelles; condensation of chromatin (i.e., nuclear condensation), as viewed by light or electron microscopy; and/or DNA cleavage into nucleosome-sized fragments, as determined by centrifuged sedimentation assays. Cell death occurs when the membrane integrity of the cell is lost (e.g., membrane

blebbing), typically, although not always, with engulfment of intact cell fragments ("apoptotic bodies") by phagocytic cells. Apoptosis differs from necrosis in which cells swell and eventually rupture. For a detailed background review of apoptosis and the cellular and biochemical events involved in the regulation of apoptosis, see, for example, Duke et al., 1996, *Sci. Am.* 275:80-87, which is incorporated herein by reference in its entirety.

Apoptosis can be determined by any suitable method, including by determining the extent of a morphological change in a cell. Such a morphological change can include, for example, progressive contraction of cell volume with the preservation of the integrity of cytoplasmic organelles and condensation of chromatin. Additionally, such a method can include determining the extent of DNA cleavage by gel electrophoresis, cell cycle analysis, or *in situ* tailing or nick translation. Additionally, such a method can include assessing membrane permeability by using dyes that bind RNA or DNA or Annexin V. Such assays for apoptosis are well known in the art; a few are described below.

For example, apoptosis of a cell can be assessed by using DNA binding dyes. These dyes are used to stain live cells for subsequent microscopic analysis or for subsequent flow cytometric analysis. When used for microscopic analysis of live cells, this technique assays nuclear morphology and membrane permeability. It provides one of the best ways of assessing apoptotic morphology in a quantitative manner and of discriminating live from dead cells and apoptotic from necrotic cells. When used for flow cytometric analysis, this technique assays membrane permeability. These dyes also bind DNA and are widely used to assay stages of the cell cycle by flow cytometry. Resting cells contain a 2N amount of DNA and form a "G1" peak, while cells in mitosis contain a 4N amount of DNA and form a 'G2' peak. DNA binding dyes have also been used at lower concentrations in fixed cells to identify an apoptotic sub-G1 peak below the resting G1 peak. This lower peak may be due to loss of DNA fragments with apoptosis or to altered conformation of apoptotic DNA. DNA laddering assays endonucleolytic cleavage of DNA into 200 base pair multimers. The scatter changes technique identifies apoptotic cells by their decreased forward scatter (due to cell shrinkage) and their increased side scatter (due to DNA condensation). This technique is easy; quantitative; can be done retrospectively on any cell sample that has been analyzed

flow cytometrically, since all samples are routinely analyzed for forward and side scatter; can be done on fresh or fixed cells, and; can be done in conjunction with cell surface phenotyping or with immunofluorescent labeling of an intracellular marker. TUNEL or *in situ* nick translation takes advantage of the classic feature of apoptosis, DNA fragmentation. The enzyme terminal deoxynucleotidyl transferase (TdT) adds labeled nucleotides to the ends of nicked DNA. The label is then revealed in different ways depending on the application; a radioactive label allows development of grains over cells in fixed tissue, while a biotin-avidin conjugate allows immunohistochemical labeling of cells in tissue or flow cytometric analysis of cells in suspension. This technique is quantitative and allows for concurrent phenotyping of cells and for analysis of expressed RNAs by *in situ* hybridization.

According to the present invention, an "apoptosis-inducing protein" or "protein that induces apoptosis" is any protein which is directly associated with inducing (e.g., causing, upregulating, initiating, propagating, increasing, or enhancing) the biological process of apoptosis in a cell. As used herein and discussed in detail below, an apoptosis-inducing protein can include a biologically active homologue of a naturally occurring apoptosis-inducing protein. Apoptosis-inducing proteins can include cellular receptors and ligands which, through ligation of such proteins, initiate apoptosis by, for example, transduction of a signal into a cell which activates other proteins in the apoptotic signal transduction pathway of the cell; and intracellular mediators of apoptotic events in the cell (e.g., DNA cleavage, condensation of chromatin), including cytoplasmic mediators involved in the formation of "death inducing complexes", apoptosis-initiating members of the caspase family which are recruited and activated by the cytoplasmic mediators, and apoptosis-effector caspases, which are recruited and activated by the initiator caspases.

Many of these apoptosis-inducing proteins are known and described in detail in the art (See, for example, the review by Duke et al., 1996, *supra*). Studies with inhibitors, dominant negative mutants of apoptosis-inducing molecules, and the phenotypes of transgenic ablated mice with defects in apoptosis-inducing molecules have elucidated two distinct pathways of initiator caspase activation in apoptosis. One pathway involves apoptosis induced by the tumor necrosis factor receptor (TNFR) family of molecules whose

cytoplasmic domains contain a "death domain" (DD). This family, which includes TNF-R1, Fas (CD95/Apo1), DR3 (Apo3), and DR4/DR5, all utilize caspase-8 as an initiator caspase. Upon crosslinking with their respective ligands (TNF, FasL, Apo3L/TWEAK and Apo2L/TRAIL), DD regions are brought into close proximity with one another initiating the formation of a "death-inducing complex" (DIC). The first molecule to bind to the clustered DD regions is a molecule called FADD (Fas-associated death domain; also called Mort-1). FADD contains a "death effector domain" (DED; also called a caspase recruitment domain or CARD) which recruits and binds the proform of caspase-8 (also called FLICE – Fas-like ICE). The pro-caspase-8 molecules recruited by the aggregated FADD molecules oligomerize and become activated through self-cleavage. Activated caspase-8 is then able to cleave the proform of the initiator caspase-9 leading to a cascade of cleavage and activation of downstream effector caspases.

The second pathway of initiator caspase activation also involves assembly of a death-inducing complex involving proteins with CARD domains. Activation of this pathway occurs through many signals including some of those that are described below. While the signals that appear to induce this pathway are quite diverse, including some that require de novo protein synthesis, mitochondria are the targets for each inducer. In brief, a very early event in this pathway involves changes in mitochondria which include release of cytochrome c, loss of mitochondrial transmembrane potential and altered redox potential. Of these, the role of cytochrome c in activation of caspases has received the greatest attention.

Cytochrome c that is released from mitochondria binds to Apaf-1, a CARD-containing, "apoptosis protease activating factor." Cytochrome c binding allows Apaf-1 molecules to self-associate and bind to the CARD domain of pro-caspase-9 forming what is called an "apoptosome". As with caspase-8 activation, activation of caspase-9 in the apoptosome is thought to occur due to aggregation of the zymogens. Pro-caspase-9 then autocleaves and initiates the downstream effector caspase activation cascade resulting in apoptosis.

According to the present invention, an "apoptosis-inhibiting protein" or "protein that inhibits apoptosis" is any protein which is directly associated with inhibiting (e.g.,

preventing, downregulating, halting, attenuating, or decreasing) the biological process of apoptosis in a cell. As used herein and discussed in detail below, an apoptosis-inhibiting protein can include a biologically active homologue of a naturally occurring apoptosis-inhibiting protein. Apoptosis-inhibiting protein include proteins that inhibit the signal transduction by apoptosis-inducing receptor/ligand interactions, proteins that inhibit the aggregation of death domains and recruitment of such proteins into death inducing complexes, proteins that inhibit the recruitment of initiator caspases, proteins that inhibit the activation of initiator caspases, and proteins that inhibit the recruitment and/or activation of effector caspases.

Many of these apoptosis-inhibiting proteins, and the mechanisms by which they act are known and described in detail in the art (See, for example, review by Duke, 1999, *ibid.*). For example, the TNFR family pathway does not require de novo protein synthesis in order to initiate apoptosis. In fact, protein synthesis inhibitors actually augment the response following receptor ligation. This appears to be due to loss of labile inhibitors. One group of such labile inhibitors are called FLIPs (FADD-like ICE inhibitory protein; also called CLARP, Casper, I-FLICE, FLAME-1, $CASH_L$ and MRIT). FLIPs have a DED/CARD domain but lack the catalytic domain of other caspases. Thus they act as decoys and either slow or block formation of the death-inducing complex. FLIPs are inactivated by a kinase called RICK (RIP-like interacting CLARP kinase) which interacts with FLIP via, not surprisingly, a DED/CARD domain.

Over-expression of members of the Bcl-2 family of proteins such as Bcl-2, Bcl-X_L, and Mcl-1 abrogate cytochrome c release and inhibit apoptosis. Members of the Bcl-2 family are anchored in the outer mitochondrial membrane, as well as in the endoplasmic reticulum and nuclear envelope, and all can form both homodimers and heterodimers with some or all of the other members, as well as with many proteins in the apoptosis-inducing pathways. In general, heterodimerization of an apoptosis-inducing protein with an apoptosis-inhibiting protein appears to abrogate the function of both proteins. In contrast, a preponderance of homodimers will shift the cell either toward or away from undergoing apoptosis.

One way that this might work is suggested by recent results studying the role of the tumor suppressor gene product Akt-1 (also called protein kinase B). Many molecules including insulin-like growth factor-1 (IGF-1) and focal adhesion kinase (FAK, see below) positively regulate Akt-1. Akt-1 appears to inhibit apoptosis by two mechanisms. First, it upregulates Bcl-X_L. Second it directly phosphorylates Bad. Phosphorylated Bad forms a complex with a molecule called 14-3-3 which prevents Bad from forming a heterodimer with either Bcl-X_L or Bcl-2, thereby allowing these molecules to block apoptosis.

A general scheme is arising which suggests that the anti-apoptotic members prevent Apaf-1 from forming a complex with caspase-9, in effect by segregating Apaf-1 away. By forming heterodimers with the anti-apoptotic members, some of the pro-apoptotic members (*e.g.*, Bik) can dislodge Apaf-1 allowing it to self-associate and participate in caspase-9 activation. The pro-apoptotic Bax molecule can act directly on mitochondria to release cytochrome c. Cytochrome c release from mitochondria can also be induced by the pro-apoptotic Bid molecule following its cleavage by active caspase-8; thereby linking the TNF-R and mitochondrial pathways.

Fig. 5 is a schematic drawing showing the points of interaction of various apoptosis-inducing proteins and apoptosis-inhibiting proteins in both the caspase-8 and the caspase-9 pathways. All such proteins are encompassed by the present invention for use in the production/propagation of a recombinant viral vector according to the present invention.

Preferred apoptosis-inducing proteins to be encoded by a recombinant viral vector according to the present invention include, but are not limited to, Fas ligand, Fas, tumor necrosis factor (TNF), tumor necrosis factor receptor (TNFR), Fas-associating death domain-containing protein (FADD), Fas-associated death domain-like IL-1β converting enzyme (FLICE), (TWEAK/Apo3L, Apo3, tumor necrosis factor-related apoptosis inducing ligand (TRAIL/Apo2L), Apo2, Bax, Bid, Bik, Bad, Bak, RICK, caspase-9, caspase-3, caspase-4, caspase-5, caspase-6, caspase-7, caspase-10, Apaf-1, cytochrome c, YAMA or biologically active homologues thereof (defined below). Members of the caspase-8 family of proteins include, but are not limited to, TNF, TNFR1, Fas ligand, Fas, Apo2, TRAIL, Apo3, TWEAK, FADD, FLICE, and caspases 3, 4, 5, 6, 7, 10, etc. Members of the caspase-9

family of proteins include, but are not limited to, cytochrome c, Apaf-1 (apoptosis protease activating protein), caspase-9 and caspases 3, 4, 5, 6, 6, 10, etc. Particularly preferred apoptosis-inducing proteins to be encoded by a recombinant viral vector according to the present invention include, but are not limited to, Fas ligand, Fas, tumor necrosis factor (TNF), Fas-associating death domain-containing protein (FADD), Fas-associated death domain-like IL-1β converting enzyme (FLICE), TWEAK/Apo3L, TRAIL/Apo2L, Bax, Bid, Bik, Bad, Bak, and RICK. The nucleic acid and amino acid sequences for these proteins in various mammalian species are known in the art, and many are disclosed herein. It is noted, however, that all of the nucleic acid and amino acid sequences of apoptosis-inducing and apoptosis-inhibiting proteins disclosed herein can be obtained from a public database such as Genbank.

A particularly preferred apoptosis-inducing protein to be encoded by a recombinant viral vector of the present invention is Fas ligand or a biologically active fragment thereof. For example, the nucleic acid sequence encoding a human Fas ligand protein is represented herein as SEQ ID NO:5. SEQ ID NO:5 encodes an amino acid sequence represented herein as SEQ ID NO:6. The nucleic acid sequence encoding a rat Fas ligand protein is represented herein as SEQ ID NO:7. SEQ ID NO:7 encodes an amino acid sequence represented herein as SEQ ID NO:8. The nucleic acid sequence encoding a mouse Fas ligand protein is represented herein as SEQ ID NO:9. SEQ ID NO:9 encodes an amino acid sequence represented herein as SEQ ID NO:10.

Nucleic acid and amino acid sequences for other apoptosis-inducing proteins discussed above are also known in the art, and a number of them are listed herein, although this list is not intended to be inclusive of sequences encompassed by the present invention. For example, the nucleic acid sequence encoding a human Fas protein is represented herein as SEQ ID NO:11. SEQ ID NO:11 encodes an amino acid sequence represented herein as SEQ ID NO:12. As discussed above, Fas (CD95/APO-1) is a transmembrane glycoprotein that is related to the receptors for tumor necrosis factor and nerve growth factor. Upon being cross-linked with agonistic anti-Fas antibodies or Fas ligand (FasL), Fas initiates a complex

signal transduction pathway that, in sensitive cell types, ultimately ends in apoptotic cell death.

The nucleic acid sequence encoding a human FADD protein is represented herein as SEQ ID NO:13. SEQ ID NO:13 encodes an amino acid sequence represented herein as SEQ ID NO:14. Fas-associating death domain-containing protein (FADD), also known as MORT1, is a cytosolic adaptor protein which is critical for signaling from CD95 (Fas) and certain other members of the tumor necrosis family. Fan et al. recently demonstrated that oligomerization of the death effector domain of FADD is sufficient to trigger apoptosis (Fan et al., 1999, *Hum. Gene Ther.* 10:2273-2285). The nucleic acid sequence encoding human FLICE is represented herein as SEQ ID NO:15. SEQ ID NO:15 encodes an amino acid sequence represented herein as SEQ ID NO:16. Fas-associated death domain-like IL-1β converting enzyme (FLICE) is also referred to as caspase-8, MACH or Mch5. FLICE is a cysteine protease that interacts with FADD and plays a critical role in the Fas signaling pathway. Nucleic acid sequences encoding other human apoptosis-inducing proteins, and the amino acid sequences encoded thereby include, but are not limited to, for example, tumor necrosis factor (TNF) (SEQ ID NO:17/SEQ ID NO:18 = nucleic acid sequence/amino acid sequence); TWEAK (SEQ ID NO:19/SEQ ID NO:20); TRAIL (SEQ ID NO:21/SEQ ID NO:22); Apo2L (SEQ ID NO:23/SEQ ID NO:24); Bax (SEQ ID NO:25/SEQ ID NO:26); Bid (SEQ ID NO:27/SEQ ID NO:28); Bik (SEQ ID NO:29/SEQ ID NO:30); Bad (SEQ ID NO:31/SEQ ID NO:32); Bak (SEQ ID NO:33/SEQ ID NO:34); and RICK (SEQ ID NO:35/SEQ ID NO:36).

In a preferred embodiment of the present invention, a recombinant viral vector of the present invention comprises a nucleic acid sequence that encodes an apoptosis-inducing protein having an amino acid sequence selected from the group of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, and biologically active homologues thereof. In another embodiment, a recombinant viral vector of the present invention comprises a nucleic acid sequence selected from the group of SEQ ID NO:5, SEQ ID NO:7,

SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, or homologues of such sequences that encode a protein having apoptosis-inducing biological activity.

Preferred apoptosis-inhibiting proteins to be encoded by a recombinant nucleic acid molecule according to the present invention include, but are not limited to, Cowpox virus caspase inhibitor (CrmA), baculovirus p35, inhibitor of apoptosis protein (IAP), dominant negative FADD, dominant negative Fas, FLIP, Bcl-2, Bcl-X_L, adenovirus E1B-19K protein, or biologically active homologues thereof (defined below). Of this list of proteins, CrmA, baculovirus p35 and IAP are inhibitors of caspase activity; dominant negative FADD, dominant negative Fas, and FLIP are inhibitors of caspase-8 activation; and Bcl-2, Bcl-X_L, and adenovirus E1B-19K protein are inhibitors of caspase-9 activation. A particularly preferred apoptosis-inhibiting protein for use in the present invention is CrmA. Nucleic acid and amino acid sequences for such apoptosis-inhibiting proteins are known in the art, and a number of them are listed herein, although this list is not intended to be inclusive of sequences encompassed by the present invention.

For example, the nucleic acid sequence encoding CrmA is represented herein as SEQ ID NO:37. SEQ ID NO:37 encodes an amino acid sequence represented herein as SEQ ID NO:38. Nucleic acid molecules encoding crmA and homologues thereof are described in PCT Publication WO 96/25501, *ibid.* Cowpox virus caspase inhibitor, or CrmA, is a protein that can inhibit Fas- and TNF-mediated apoptosis. Nucleic acid sequences encoding other apoptosis-inhibiting proteins, and the amino acid sequences encoded thereby include, but are not limited to, for example, human inhibitor of apoptosis protein (IAP) (SEQ ID NO:39/SEQ ID NO:40 = nucleic acid sequence/amino acid sequence); human dominant negative Fas (SEQ ID NO:41/SEQ ID NO:42; or alternatively, a truncated naturally occurring Fas where at least a portion of the "death domain" from nucleotide positions 1029-1199 of SEQ ID NO:11 or amino acid positions 279-335 of SEQ ID NO:12 are removed); human FLIP (SEQ ID NO:43/SEQ ID NO:44); human Bcl-2 (SEQ ID NO:45/SEQ ID NO:46); human Bcl-X_L (SEQ ID NO:47/SEQ ID NO:48); adenovirus E1B-19K (SEQ ID NO:49/SEQ ID NO:50)

and human dominant negative FADD (truncated version of naturally occurring FADD comprising nucleotide positions 367-753 of SEQ ID NO:13 and amino acid positions 80-208 of SEQ ID NO:14).

In a preferred embodiment of the present invention, a recombinant nucleic acid molecule comprising a nucleic acid sequence that encodes an apoptosis-inhibiting protein encodes a protein comprising an amino acid sequence selected from the group of SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, amino acid positions 80-208 of SEQ ID NO:14, and biologically active homologues thereof. In another embodiment, a recombinant nucleic acid molecule comprising a nucleic acid sequence encoding an apoptosis-inhibiting protein is selected from the group of SEQ ID NO:37, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, nucleotide positions 367-753 of SEQ ID NO:13, or homologues of such sequences that encode a protein having apoptosis-inducing biological activity.

According to the present invention, an isolated, or biologically pure, protein, is a protein that has been removed from its natural milieu. As such, "isolated" and "biologically pure" do not necessarily reflect the extent to which the protein has been purified. An isolated protein, as used herein with regard to apoptosis-inhibiting proteins and/or apoptosis-inducing proteins, is preferably produced using recombinant DNA technology. As used herein, reference to a particular protein (e.g., an apoptosis-inducing protein or an apoptosis-inhibiting protein) includes a full-length protein or any homologue of such a protein. Protein homologues are variants of a naturally occurring protein in which at least one or a few, but not limited to one or a few, amino acids have been deleted (e.g., a truncated version of the protein, such as a peptide), inserted, inverted, substituted and/or derivatized (e.g., by glycosylation, phosphorylation, acetylation, myristylation, prenylation, palmitoylation, amidation and/or addition of glycerophosphatidyl inositol) such that the homologue includes at least one epitope capable of eliciting an immune response against the corresponding naturally occurring protein and/or has a biological activity of the corresponding naturally occurring protein. To be "capable of eliciting an immune response" indicates that when the

homologue is administered to an animal as an immunogen, using techniques known to those skilled in the art, the animal will produce a humoral and/or cellular immune response against at least one epitope of the naturally occurring protein. To have "biological activity of the naturally occurring protein" indicates that the protein is sufficiently structurally similar to the corresponding naturally occurring protein such that the protein has biological activity substantially similar to the naturally occurring protein. The biological activity of a protein can include the ability of a protein to bind to another protein or molecule, to activate another molecule, to become activated, and/or to perform the naturally occurring function of the protein in a cell or biological system. The biological activity of the apoptosis-inducing and apoptosis-inhibiting proteins disclosed herein can be measured using methods known in the art. For example, CrmA biological activity can be measured by its ability to inhibit Fas-induced or TNF-induced apoptosis in an *in vitro* assay, for example, as described in PCT Publication No. WO 96/25501, which is incorporated herein by reference in its entirety. It is to be understood that the biological activity of a homologue of the preset invention is a qualitative, rather than a quantitative, characteristic, in that a protein homologue having the biological activity of a naturally occurring protein function as the naturally occurring protein (e.g., inhibiting or inducing apoptosis in a cell), but can do so at a greater, equal or lesser measurable degree than the naturally occurring protein.

In one embodiment, the minimal size of a protein homologue of the present invention is a size sufficient to be encoded by a nucleic acid molecule capable of forming a stable hybrid with the complementary sequence of a nucleic acid molecule encoding the corresponding natural protein. As such, the size of the nucleic acid molecule encoding such a protein homologue is dependent on nucleic acid composition and percent homology between the nucleic acid molecule and complementary sequence as well as upon hybridization conditions per se (e.g., temperature, salt concentration, and formamide concentration). The minimal size of such nucleic acid molecules is typically at least about 12 to about 15 nucleotides in length if the nucleic acid molecules are GC-rich and at least about 15 to about 17 bases in length if they are AT-rich. As such, the minimal size of a nucleic acid molecule used to encode a protease protein homologue of the present invention

is from about 12 to about 18 nucleotides in length. There is no limit, other than a practical limit, on the maximal size of such a nucleic acid molecule in that the nucleic acid molecule can include a portion of a gene, an entire gene, or multiple genes, or portions thereof. Similarly, the minimal size of a protease protein homologue of the present invention is from about 4 to about 6 amino acids in length, with preferred sizes depending on whether a full-length, multivalent (i.e., fusion protein having more than one domain each of which has a function), or functional portions of such proteins are desired. Apoptosis-inducing protein homologues of the present invention have the ability to induce apoptosis in a cell under suitable conditions as described herein and/or are capable of eliciting an immune response against a naturally occurring apoptosis-inducing protein. Apoptosis-inhibiting protein homologues of the present invention have the ability to inhibit apoptosis in a cell under suitable conditions as described herein and/or are capable of eliciting an immune response against a naturally occurring apoptosis-inhibiting protein.

In one embodiment, a homologue of an apoptosis-inducing or apoptosis-inhibiting protein of the present invention comprises an amino acid sequence comprising at least about 6, and more preferably at least about 12 and more preferably at least about 24 contiguous amino acid residues of an amino acid sequence of a naturally occurring (i.e., wild-type) protein. In another embodiment, a homologue is encoded by a nucleic acid sequence comprising at least about 18, and more preferably at least about 36, and even more preferably at least about 72 contiguous nucleotides of a nucleic acid sequence encoding a naturally occurring protein.

Protein homologues can be the result of natural allelic variation or natural mutation. According to the present invention, protein homologues can also be produced using techniques known in the art including, but not limited to, direct modifications to the protein or modifications to the gene encoding the protein using, for example, classic or recombinant DNA techniques to effect random or targeted mutagenesis.

It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, a gene refers to one or more genes or at least one gene. As such, the terms "a"

(or "an"), "one or more" and "at least one" can be used interchangeably herein. It is also to be noted that the terms "comprising", "including", and "having" can be used interchangeably.

In accordance with the present invention, an isolated nucleic acid molecule is a nucleic acid molecule that has been removed from its natural milieu (i.e., that has been subject to human manipulation). As such, "isolated" does not reflect the extent to which the nucleic acid molecule has been purified. An isolated nucleic acid molecule can include DNA, RNA, or derivatives of either DNA or RNA. According to the present invention, a nucleic acid molecule encoding a given protein (e.g., a Fas ligand protein) can include an isolated natural gene, a portion of such a gene or a homologue thereof, the latter of which is described in more detail below. As used herein, a nucleic acid molecule can include one or more regulatory regions, full-length or partial coding regions, or combinations thereof. The minimal size of such a nucleic acid molecule is the minimal size that can form a stable hybrid with a naturally occurring gene under stringent hybridization conditions. An isolated nucleic acid molecule can be obtained from its natural source either as an entire (i.e., complete) gene or a portion thereof capable of forming a stable hybrid with that gene. An isolated nucleic acid molecule can also be produced using recombinant DNA technology (e.g., polymerase chain reaction (PCR) amplification, cloning) or chemical synthesis. Isolated nucleic acid molecules include natural nucleic acid molecules and homologues thereof, including, but not limited to, natural allelic variants and modified nucleic acid molecules in which nucleotides have been inserted, deleted, substituted, and/or inverted in such a manner that such modifications do not substantially interfere with the nucleic acid molecule's ability to encode the desired protein which is useful in the present invention or to form stable hybrids under stringent conditions with natural gene isolates.

As used herein, stringent hybridization conditions refer to standard hybridization conditions under which nucleic acid molecules are used to identify similar nucleic acid molecules. Such standard conditions are disclosed, for example, in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labs Press, 1989. Sambrook et al., *ibid.*, is incorporated by reference herein in its entirety (see specifically, pages 9.31-9.62). In addition, formulae to calculate the appropriate hybridization and wash conditions

to achieve hybridization permitting varying degrees of mismatch of nucleotides are disclosed, for example, in Meinkoth et al., 1984, *Anal. Biochem.* 138, 267-284; Meinkoth et al., *ibid.*, is incorporated by reference herein in its entirety.

More particularly, stringent hybridization and washing conditions, as referred to herein, refer to conditions which permit isolation of nucleic acid molecules having at least about 70% nucleic acid sequence identity with the nucleic acid molecule being used to probe in the hybridization reaction, more particularly at least about 75%, and most particularly at least about 80%. Such conditions will vary, depending on whether DNA:RNA or DNA:DNA hybrids are being formed. Calculated melting temperatures for DNA:DNA hybrids are 10°C less than for DNA:RNA hybrids. In particular embodiments, stringent hybridization conditions for DNA:DNA hybrids include hybridization at an ionic strength of 6X SSC (0.9 M Na^+) at a temperature of between about 20°C and about 35°C, more preferably, between about 28°C and about 40°C, and even more preferably, between about 35°C and about 45°C. In particular embodiments, stringent hybridization conditions for DNA:RNA hybrids include hybridization at an ionic strength of 6X SSC (0.9 M Na^+) at a temperature of between about 30°C and about 45°C, more preferably, between about 38°C and about 50°C, and even more preferably, between about 45°C and about 55°C. These values are based on calculations of a melting temperature for molecules larger than about 100 nucleotides, 0% formamide and a G + C content of about 40%. Alternatively, T_m can be calculated empirically as set forth in Sambrook et al., *supra*, pages 9.31 TO 9.62.

A nucleic acid molecule homologue can be produced using a number of methods known to those skilled in the art (see, for example, Sambrook et al., *ibid.*). For example, nucleic acid molecules can be modified using a variety of techniques including, but not limited to, classic mutagenesis techniques and recombinant DNA techniques, such as site-directed mutagenesis, chemical treatment of a nucleic acid molecule to induce mutations, restriction enzyme cleavage of a nucleic acid fragment, ligation of nucleic acid fragments, PCR amplification and/or mutagenesis of selected regions of a nucleic acid sequence, synthesis of oligonucleotide mixtures and ligation of mixture groups to "build" a mixture of nucleic acid molecules and combinations thereof. Nucleic acid molecule homologues can

be selected from a mixture of modified nucleic acids by screening for the function of the protein encoded by the nucleic acid (e.g., ability to elicit an immune response against at least one epitope of the naturally occurring protein, ability to selectively bind to immune serum, ability to bind to glutathione), by hybridization with the naturally occurring gene, and/or by the desired biological activity.

The present invention also includes nucleic acid molecules encoding an apoptosis-inhibiting protein or an apoptosis-inducing protein that have been modified to accommodate codon usage properties of the cells in which such nucleic acid molecules are to be expressed.

Knowing the nucleic acid sequences of certain nucleic acid molecules useful in the present invention allows one skilled in the art to, for example, (a) make copies of those nucleic acid molecules, (b) obtain nucleic acid molecules including at least a portion of such nucleic acid molecules (e.g., nucleic acid molecules including full-length genes, full-length coding regions, regulatory control sequences, truncated coding regions), and (c) obtain similar nucleic acid molecules from other mammals, particularly since, as described in detail in the Examples section, knowledge of a nucleic acid molecule from one species (e.g., rat Fas ligand) enables the isolation of the corresponding nucleic acid molecule from another species (e.g., human Fas ligand). Such nucleic acid molecules can be obtained in a variety of ways including screening appropriate expression libraries with antibodies against the protein encoded by the desired nucleic acid molecule; traditional cloning techniques using oligonucleotide probes, such as those of the present invention for Fas ligand, to screen appropriate libraries or DNA; and PCR amplification of appropriate libraries or DNA using oligonucleotide primers.

The present invention also includes a recombinant vector, which can be any vector capable of enabling recombinant production of a protein and/or which can deliver the nucleic acid molecule into a host cell. Such a vector contains heterologous nucleic acid sequences (e.g., nucleic acid sequences that are not naturally found adjacent to the nucleic acid molecule to be expressed). The vector can be either RNA or DNA, either prokaryotic or eukaryotic, and typically is a virus or a plasmid. Recombinant vectors can be used in the cloning, sequencing, and/or otherwise manipulating of nucleic acid molecules useful in the

present invention. Preferred recombinant vectors are capable of replicating when transformed into a suitable host cell. As used herein, a recombinant vector which is linked to a nucleic acid sequence encoding the desired protein (e.g., an apoptosis-inhibiting protein or an apoptosis-inducing protein) can be referred to herein as either a recombinant vector or a recombinant molecule. Recombinant viral vectors of the present invention are described in detail below.

A recombinant molecule of the present invention is a molecule that can include at least one of any nucleic acid molecule encoding an apoptosis-inhibiting protein or a nucleic acid molecule encoding an apoptosis-inducing protein operatively linked to at least one of any transcription control sequence capable of effectively regulating expression of the nucleic acid molecule(s) in the cell to be transformed, examples of which are disclosed herein. Although the phrase "nucleic acid molecule" primarily refers to the physical nucleic acid molecule and the phrase "nucleic acid sequence" primarily refers to the sequence of nucleotides on the nucleic acid molecule, the two phrases can be used interchangeably, especially with respect to a nucleic acid molecule, or a nucleic acid sequence, being capable of encoding a protein. In addition, the phrase "recombinant molecule" primarily refers to a nucleic acid molecule operatively linked to a transcription control sequence, but can be used interchangeably with the phrase "nucleic acid molecule" which is administered to an animal.

The recombinant nucleic acid molecule comprising a nucleic acid sequence encoding an apoptosis-inhibiting protein and the recombinant viral vector comprising a nucleic acid sequence encoding an apoptosis-inducing protein can either be separate molecules, both of which are introduced into the production cell line, or alternatively, the recombinant nucleic acid molecule comprising a nucleic acid sequence encoding an apoptosis-inhibiting protein can be contained within (i.e., is a portion of) the recombinant viral vector that comprises a nucleic acid sequence encoding an apoptosis-inducing protein. In this latter instance, the nucleic acid sequence encoding an apoptosis-inhibiting protein can be operatively linked to either the same transcription control sequence as the nucleic acid sequence encoding an apoptosis-inducing protein, or to a different transcription control sequence. In one embodiment, when the two nucleic acid sequences are operatively linked to the same

transcription control sequence, the nucleic acid sequence encoding an apoptosis-inhibiting protein and the nucleic acid sequence encoding an apoptosis-inducing protein are separated in the recombinant viral vector by an internal ribosome entry site (IRES) (described in detail below).

Therefore, one embodiment of the present invention relates to a recombinant viral vector for inducing apoptosis in cells infected by the vector. The vector includes: (a) a nucleic acid sequence encoding an apoptosis-inhibiting protein operatively linked to a transcription control sequence and (b) a nucleic acid sequence encoding an apoptosis-inducing protein operatively linked to a transcription control sequence. The nucleic acid sequences of (a) and (b) can be operatively linked to the same or different transcription control sequences, and when linked to the same transcription control sequence, are preferably separated by an IRES.

In a recombinant molecule of the present invention, including a recombinant viral vector, nucleic acid molecules are operatively linked to expression vectors containing regulatory sequences such as transcription control sequences, translation control sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant cell and that control the expression of nucleic acid molecules of the present invention and/or the propagation of the recombinant viral vector, when applicable. In particular, recombinant molecules of the present invention include nucleic acid sequences that are operatively linked to one or more transcription control sequences. The phrase "operatively linked" refers to linking a nucleic acid sequence to a transcription control sequence in a manner such that the molecule is able to be expressed when transfected (i.e., transformed, transduced, infected or transfected) into a host cell. Transcription control sequences are sequences which control the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. Suitable transcription control sequences include any transcription control sequence that can function in at least one of the recombinant cells of the present invention. A variety of such transcription control sequences are known to those skilled in the art. Preferred transcription

control sequences include those which function in bacterial, yeast, insect and mammalian cells, such as, but not limited to, *tac*, *lac*, *trp*, *trc*, oxy-pro, omp/lpp, rrnB, bacteriophage lambda (λ) (such as λp_L and λp_R and fusions that include such promoters), bacteriophage T7, T7*lac*, bacteriophage T3, bacteriophage SP6, bacteriophage SP01, metallothionein, α-mating factor, *Pichia* alcohol oxidase, alphavirus subgenomic promoters (such as Sindbis virus subgenomic promoters), antibiotic resistance gene, baculovirus, *Heliothis zea* insect virus, vaccinia virus, herpesvirus, raccoon poxvirus, other poxvirus, adenovirus, cytomegalovirus (such as intermediate early promoters, simian virus 40, retrovirus, actin, retroviral long terminal repeat, Rous sarcoma virus, heat shock, phosphate and nitrate transcription control sequences as well as other sequences capable of controlling gene expression in prokaryotic or eukaryotic cells. Particularly preferred transcription control sequences include tissue-specific promoters (e.g., insulin promoters, α-myosin heavy chain promoter and endothelin promoter) and enhancers, as well as lymphokine-inducible promoters (e.g., promoters inducible by interferons or interleukins) and constitutively active promoters (e.g., β-activin promoter and ubiquitin promoter). Transcription control sequences of the present invention can also include naturally occurring transcription control sequences naturally associated with the protein to be expressed prior to isolation.

According to the present invention, a recombinant molecule can be dicistronic. A cistron refers to a unit of DNA that is capable of encoding an amino acid sequence having a naturally-occurring biological function. A dicistronic plasmid refers to a plasmid containing two cistrons. Preferably, a dicistronic recombinant molecule of the present invention comprises an internal ribosome entry site (IRES) element to which eukaryotic ribosomes can bind (see, for example, Jang et al., *J. Virol.* 62:2636-2643, 1988; Pelletier et al. *Nature* 334:320-325, 1988; Jackson, *Nature* 353:14-15, 1991; Macejek et al., *Nature* 353:90-94, 1991; Oh et al., *Genes & Develop.* 6:1643-1653, 1992; Molla et al., *Nature* 356:255-257, 1992; and Kozak, *Crit. Rev. Biochem. Molec. Biol.* 27(4,5):385-402, 1992).

Recombinant molecules of the present invention may also (a) contain secretory signals (i.e., signal segment nucleic acid sequences) to enable an expressed protein to be secreted from the cell that produces the protein and/or (b) contain fusion sequences which

lead to the expression of nucleic acid molecules as fusion proteins. Eukaryotic recombinant molecules may include intervening and/or untranslated sequences surrounding and/or within the nucleic acid sequences of nucleic acid molecules. Suitable signal segments include any signal segment capable of directing the secretion of a given protein. Preferred signal segments include, but are not limited to, tissue plasminogen activator (t-PA), interferon, interleukin, growth hormone, histocompatibility and viral envelope glycoprotein signal segments.

The method of the present invention is used for propagation of a recombinant viral vector comprising a nucleic acid sequence encoding an apoptosis-inducing protein. Such a vector includes a recombinant molecule of the present invention that is packaged in a viral coat and that can be expressed in an animal or recombinant cell after administration. Preferably, the viral vector is capable of being propagated (e.g., replicated and packaged) when introduced into an appropriate host cell (e.g., a production cell line). In one embodiment, the recombinant viral vector is packaging- and/or replication-deficient. A number of recombinant viral vectors can be used, including, but not limited to, those based on alphaviruses, poxviruses, adenoviruses, herpesviruses, lentiviruses, adeno-associated viruses, vaccinia viruses, baculoviruses, parvoviruses and retroviruses. Particularly preferred viral vectors are those based on adenoviruses, adeno-associated viruses and lentiviruses. When introduced into a production cell line that is capable of replicating and packaging the viral vector (e.g., a cell that has been transformed with or naturally carries viral genes required for replication and/or packaging of a virus from which the viral vector is derived), the recombinant viral vector is propagated. Moreover, when infected into a recipient host cell under the appropriate conditions according to the present invention, a recombinant viral vector of the present invention can direct the production of the encoded apoptosis-inducing protein. Viral vectors suitable for gene delivery are well known in the art and can be selected by the skilled artisan for use in the present invention. A detailed discussion of current viral vectors is provided in "Molecular Biotechnology," Second Edition, by Glick and Pasternak, ASM Press, Washington D.C., 1998, pp. 555-590, the entirety of which is incorporated herein by reference. One viral vector for use in the present invention is a human adenovirus

5 construct under the control of a CMV immediate early promoter. A viral vector encoding a Fas ligand protein is described in detail in the Examples section, and comprises a nucleic acid sequence that comprises at least a portion of a nucleic acid sequence represented herein by SEQ ID NO:4. Referring to SEQ ID NO:4, the CMV immediate early promoter, coding sequence, SV40 small t intron and SV40 polyA can be inserted between nucleotides 454 and 455. Therefore, a viral vector encoding Fas ligand protein can include all or a sufficient portion of SEQ ID NO:4 (e.g., sufficient to produce a recombinant viral vector according to the present invention), with the promoter, coding sequence and other required regulatory sequences, such as the SV40 sequences referenced above, inserted at the above-designated position.

An adenoviral vector is one preferred vector for use in the present methods. An adenoviral vector infects a wide range of nondividing human cells and has been used extensively in live vaccines without adverse side effects. Adenoviral vectors do not integrate into the host genome, and therefore, gene therapy using this system requires periodic administration, although methods have been described which extend the expression time of adenoviral transferred genes, such as administration of antibodies directed against T cell receptors at the site of expression (Sawchuk et al., 1996, *Hum. Gene. Ther.* 7:499-506). It is noted, however, that for use in the therapeutic methods of the present invention as described below, it is typically not necessary that the expression of the apoptosis-inducing protein by the viral vector be long-term, and in fact, short term expression is typically preferred. More particularly, expression of an apoptosis-inducing protein in the methods of the present invention (e.g., for the induction of tolerance and/or elimination of targeted host lymphocytes or for cancer therapy) is preferably accomplished by short term exposure of the target cells to the protein. Such short term exposure is sufficient to be effective for induction of apoptosis in a target cell (and/or tolerance, in the case of lymphocytes), and minimizes potential undesirable side effects of having an apoptosis-inducing protein available in a patient, which would increase with long-term expression.

The efficiency of adenovirus-mediated gene delivery can be enhanced by developing a virus that preferentially infects a particular target cell. For example, a gene for the

attachment fibers of adenovirus can be engineered to include a DNA element that encodes a protein domain that binds to a cell-specific receptor. Examples of successful *in vivo* delivery of genes has been demonstrated and are discussed in more detail below.

Yet another type of viral vector is based on adeno-associated viruses, which are small, nonpathogenic, single-stranded human viruses. This virus can integrate into a specific site on chromosome 19. This virus can carry a cloned insert of about 4.5 kb, and has typically been successfully used to express proteins *in vivo* from 70 days to at least 5 months. Demonstrating that the art is quickly advancing in the area of gene therapy, however, a recent publication by Bennett et al. reported efficient and stable transgene expression by adeno-associated viral vector transfer *in vivo* for greater than 1 year (Bennett et al., 1999, *Proc. Natl. Acad. Sci. USA* 96:9920-9925). As discussed above, however, the methods of the present invention do not typically require long-term expression, and so any of the viral vectors described herein would be sufficient for use in the therapeutic methods of the present invention.

When it is desired to have a nucleic acid sequence inserted into the host genome for long term expression, a retroviral vector can be packaged in the envelope protein of another virus so that it has the binding specificity and infection spectrum that are determined by the envelope protein (e.g., a pseudotyped virus). In addition, the envelope gene can be genetically engineered to include a DNA element that encodes and amino acid sequence that binds to a cell receptor to create a recombinant retrovirus that infects a specific cell type. Expression of the gene (e.g., a Fas ligand gene) can be further controlled by the use of a cell or tissue-specific promoter. Retroviral vectors have been successfully used to transfect cells with a gene which is expressed and maintained in a variety of *ex vivo* systems.

It may be appreciated by one skilled in the art that use of recombinant DNA technologies can improve expression of transformed nucleic acid molecules by manipulating, for example, the number of copies of the nucleic acid molecules within a host cell, the efficiency with which those nucleic acid molecules are transcribed, the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications. Recombinant techniques useful for increasing the expression of nucleic acid molecules of

the present invention include, but are not limited to, operatively linking nucleic acid molecules to high-copy number plasmids, integration of the nucleic acid molecules into one or more host cell chromosomes, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals (e.g., Shine-Dalgarno sequences), modification of nucleic acid molecules to correspond to the codon usage of the host cell, deletion of sequences that destabilize transcripts, and use of control signals that temporally separate recombinant cell growth from recombinant enzyme production during fermentation. The activity of an expressed recombinant protein of the present invention may be improved by fragmenting, modifying, or derivatizing nucleic acid molecules encoding such a protein.

According to the method of the present invention, a recombinant viral vector comprising a nucleic acid molecule encoding an apoptosis-inducing protein can be propagated by culturing a production cell line, also referred to generally as an isolated cell, that has been transfected with a recombinant nucleic acid molecule encoding an apoptosis-inducing protein operatively linked to a transcription control sequence, and with a recombinant viral vector comprising a recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a protein that induces apoptosis in a cell. An isolated cell transfected with a recombinant viral vector and/or other recombinant nucleic acid molecule can also generally be referred to as a recombinant cell. As used herein, an isolated cell which is used to propagate a recombinant viral vector, can also be referred to as a production cell or cell line, and an isolated cell that is used to produce an apoptosis-inducing protein (such as in a therapeutic method of the present invention) can be referred to as a host cell. In some instances, a production cell and a host cell are the same cell. For example, the production cell line can also be capable of expressing the apoptosis-inducing protein under conditions effective to produce the protein, if desired (e.g., when administered to a patient to suppress graft rejection). Alternatively, the propagated viral vector can be recovered from the production cell line and transfected into another suitable host cell where the apoptosis-inducing protein can be expressed. An isolated cell that is used to carry a recombinant vector

or molecule to an appropriate *in vitro* or *in vivo* site can also be referred to as a pharmaceutically acceptable carrier. Transfection of a recombinant nucleic acid molecule or a recombinant viral vector into a cell can be accomplished by any method by which a nucleic acid molecule can be inserted into the cell. Transfection techniques include, but are not limited to, transformation, electroporation, microinjection, lipofection, adsorption, infection and protoplast fusion. In the case of a recombinant viral vector, the host cell is preferably transfected by infection.

A host cell that has been transfected with a recombinant nucleic acid molecule and/or a recombinant viral vector can be referred to herein as a recombinant cell. A recombinant cell may remain unicellular or may grow into a tissue, organ or a multicellular organism. Preferably, a recombinant cell of the present invention is suitable for propagating the recombinant viral vector encoding an apoptosis-inducing protein. Such a cell is typically referred to herein as a production cell, and for some viral vectors, is referred to as a packaging cell line. Transfected nucleic acid molecules of the present invention can remain extrachromosomal or can integrate into one or more sites within a chromosome of the transfected (i.e., recombinant) cell in such a manner that their ability to be expressed is retained.

Suitable cells to transfect for the method of propagating a viral vector according to the present invention include any isolated cell that can be transfected with a recombinant nucleic acid molecule and a recombinant viral vector, and which can replicate the viral vector. For example, packaging cell lines are specialized cells in that they have additionally been transfected with or naturally contain viral genes which allow the transfected viral vector to be propagated. Isolated production cells can be either untransfected cells or cells that are already transfected with at least one nucleic acid molecule. Isolated cells of the present invention can be any cell capable of producing a recombinant protein and/or capable of propagating a recombinant viral vector and include mammalian, bacterial, fungal (including yeast), parasite, insect, other animal and plant cells. In one embodiment of the present invention, preferred production cells include bacterial, insect and mammalian cells. Particularly preferred production cells include embryonal kidney cells, such as the 293 cell

line described in the Examples section. 293 (ATCC Accession No. CRL-1573) has been transformed by the E1 region of the adenovirus 5 chromosome and is therefore useful for propagating viral vectors derived from adenoviruses.

In one embodiment of the present invention, and particularly, therapeutic methods of the present invention, a suitable cell to be transfected with a recombinant viral vector encoding an apoptosis-inducing protein includes host cells (e.g., cells which are to be the final recipient of or the carrier of the recombinant viral vector), such as any mammalian cells which are useful and desirable as a transplantable graft (e.g., an insulin-producing beta cell). Such a host cell can also be a part of an organ which is to be transplanted (e.g., a kidney having multiple cells transformed with Fas ligand). Additionally, such a host cell can be any cell which is transplanted in conjunction with another non-transformed cell or organ (i.e., a fibroblast cell which has been transformed to express Fas ligand that is transplanted with a non-transformed beta cell or a kidney comprising non-transformed cells). In certain embodiments, discussed below, such a host cell can also be transfected with a recombinant nucleic acid molecule encoding an apoptosis-inhibiting protein as described herein.

Effective conditions for propagation of a recombinant viral vector and/or effective conditions to produce an apoptosis-inducing protein include, but are not limited to, appropriate media, bioreactor, temperature, pH and oxygen conditions that permit viral propagation and/or protein production. Similarly, effective conditions for induction of apoptosis in a cell include, but are not limited to, the presence of a required cofactor or receptor for induction of apoptosis, and culture or environmental conditions which allow apoptosis to occur. An effective medium is typically an aqueous medium comprising assimilable carbon, nitrogen and phosphate sources, as well as appropriate salts, minerals, metals and other nutrients, such as vitamins. The medium may comprise complex nutrients or may be a defined minimal medium. Cells can be cultured in conventional fermentation bioreactors, which include, but are not limited to, batch, fed-batch, cell recycle, and continuous fermentors. Culturing can also be conducted in shake flasks, test tubes, microtiter dishes, and petri plates. Culturing is carried out at a temperature, pH and oxygen content appropriate for the isolated cell. Such culturing conditions are well within the expertise of

one of ordinary skill in the art. Examples of suitable conditions are included in the Examples section.

Depending on the vector and host system used for production, resultant recombinantly produced proteins may either remain within the isolated cell (i.e., be expressed internally); be secreted into the culture medium; be secreted into a space between two cellular membranes, such as the periplasmic space in *E. coli*; or be retained on the outer surface of a cell or viral membrane.

The phrase "recovering the viral vector" or "recovering the protein" refers simply to collecting the whole culture medium containing the viral vector and/or protein and need not imply additional steps of separation or purification. Recombinant viral vectors can be recovered from production cells using a variety of standard techniques, including but not limited to lysis, centrifugation, and chromatography. Apoptosis-inducing proteins can be purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization.

Using the production method of the present invention, a viral vector encoding an apoptosis-inducing protein according to the present invention can be replicated to a viral titer of at least about 1×10^8 pfu per ml of supernatant isolated from the cell (i.e., using standard methods for isolating virus from a production cell line) and more preferably at least about 5×10^8 pfu per ml of supernatant isolated from the cell, and even more preferably at least about 1×10^9 pfu per ml of supernatant isolated from the cell, and even more preferably at least about 5×10^9 pfu per ml of supernatant isolated from the cell, and even more preferably at least about 1×10^{10} pfu per ml of supernatant isolated from the cell, without damaging or destroying the production cell (i.e., the packaging cell line).

The recombinant viral vector of the present invention, isolated cells transfected with such a recombinant viral vector, and the method of propagating such a recombinant viral vector, are useful in embodiments of the present invention related to therapeutic methods of

preventing and treating diseases and conditions including graft rejection, cancer and T-lymphocyte mediated diseases. Accordingly, another embodiment of the present invention relates to a method of suppressing T-lymphocyte-mediated graft rejection in a recipient mammal. The method comprises introducing into the mammal a pharmaceutically acceptable carrier comprising a recombinant viral vector comprising a nucleic acid sequence encoding a protein that induces apoptosis operatively linked to a transcription control sequence. In one embodiment of this method, the pharmaceutically acceptable carrier can additionally comprise a recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a protein that inhibits apoptosis operatively linked to a transcription control sequence. In this embodiment, the nucleic acid sequence encoding the apoptosis-inhibiting protein is preferably contained within the recombinant viral vector, although the recombinant nucleic acid molecule can be administered as a separate molecule, particularly when delivered to a cell by *ex vivo* methods. When the nucleic acid sequence encoding the apoptosis-inhibiting protein is within the recombinant viral vector, the nucleic acid sequence encoding this protein and the nucleic acid sequence encoding the apoptosis-inducing protein can be operatively linked to the same or different transcription control sequences, and when linked to the same transcription control sequence, are preferably separated by an IRES. Such vectors, recombinant molecules, apoptosis-inducing proteins and apoptosis-inhibiting proteins have been described above. In one embodiment, the apoptosis-inducing protein is Fas ligand.

In one embodiment, the pharmaceutically acceptable carrier is an isolated cell that is transfected with the recombinant viral vector, alone or in combination with the recombinant nucleic acid molecule encoding an apoptosis-inhibiting protein. In this embodiment, the cell preferably has the characteristics of a suitable host cell of the present invention as described above. Specifically, the cellular carrier should be capable of expressing the apoptosis-inducing protein and, when included, the apoptosis-inhibiting protein. According to the present invention, the apoptosis-inhibiting protein is included in the pharmaceutically acceptable carrier when it is desirable to protect the carrier cell from undergoing apoptosis. In one embodiment, the cellular carrier is capable of expressing the

apoptosis-inducing protein at the site of transplantation. In one embodiment, the carrier cell is a part of a graft to be transplanted in a recipient, although the cell does not necessarily need to be a part of the graft (e.g., the cell can be a heterologous cell that is associated with the graft *ex vivo* for the purposes of providing an apoptosis-inducing protein to protect the graft). In the former embodiment, the transplanted tissue itself functions as a source of an apoptosis-inducing protein, which in a preferred embodiment, is Fas ligand. In this embodiment, transplanted tissue is obtained from a non-human animal is transfected with the recombinant viral vector encoding the apoptosis-inducing protein and in some embodiments, with a recombinant nucleic acid molecule encoding an apoptosis-inhibiting protein. As discussed above, the recombinant nucleic acid molecule encoding an apoptosis-inhibiting protein can be contained within the recombinant viral vector, or can be transfected as a separate nucleic acid molecule. The transfected cells maintain their ability to express biologically active apoptosis-inducing protein when transplanted into the recipient host animal. The invention includes the transplant of apoptosis-inducing protein-expressing tissue alone (e.g., transplant of transgenic islet cells into a diabetic patient) or transplantation of apoptosis-inducing protein-expressing autologous tissue along with non-manipulated donor tissue (e.g., transplant of a non-transgenic islet cells to a patient in need thereof with apoptosis-inducing protein-expressing tissue to the graft site, thereby creating an artificial immunologically-privileged site). In this latter case, the transplanted apoptosis-inducing protein-expressing tissue functions to suppress rejection of the transplanted islet cells. Preferred methods of administering such a pharmaceutically acceptable carrier are discussed in detail below.

According to the present invention, donor tissue may he obtained from the same or a different species as the recipient mammal. The term "donor tissue", includes cells and organs from a donor mammal, including but not limited to islet cells, kidney, heart, liver, lung, brain, and muscle tissue. In one embodiment, the donor tissue may be obtained from any mammal, and preferably pigs. Pigs offer many advantages for use as organ and cell donor animals. For example, many porcine organs, such as the heart and kidney, are of a similar size to human organs. In another embodiment of the present invention, allogeneic cells (i.e., cells derived from a source other than a patient, but that are histotype compatible

with the patient) or autologous cells (i.e., cells isolated from a patient) are transfected with recombinant viral vector described herein. Such cells can then be referred to as a portion of a therapeutic composition for suppressing graft rejection in the mammal. Such a therapeutic composition is then administered to a patient by any suitable means of administration, including, but not limited to, intradermal, intravenous or subcutaneous injection, or direct injection at the site of transplantation during transplant surgery.

In one embodiment, the pharmaceutically acceptable carrier can include a liposome which contains the recombinant viral vector and delivers the vector to a suitable site in a host recipient. According to the present invention, a liposome pharmaceutically acceptable carrier comprises a lipid composition that is capable of delivering a recombinant nucleic acid molecule or viral vector of the present invention to a suitable cell and/or tissue in a mammal. A liposome pharmaceutically acceptable carrier of the present invention comprises a lipid composition that is capable of fusing with the plasma membrane of the target cell to deliver the recombinant nucleic acid molecule and/or viral vector into a cell. A liposome pharmaceutically acceptable carrier of the present invention can be modified to target a particular site in a mammal (i.e., a targeting liposome), such as the site of transplantation, thereby targeting and making use of a nucleic acid molecule of the present invention at that site. Suitable modifications include manipulating the chemical formula of the lipid portion of the pharmaceutically acceptable carrier. Manipulating the chemical formula of the lipid portion of the pharmaceutically acceptable carrier can elicit the extracellular or intracellular targeting of the pharmaceutically acceptable carrier. For example, a chemical can be added to the lipid formula of a liposome that alters the charge of the lipid bilayer of the liposome so that the liposome fuses with particular cells having particular charge characteristics. Other targeting mechanisms include targeting a site by addition of exogenous targeting molecules to a liposome such as an antibody, soluble receptor or ligand, incorporated with the liposome, to target a particular cell or tissue to which the targeting molecule can bind. Targeting liposomes are described, for example, in Ho et al., 1986, *Biochemistry* 25: 5500-6; Ho et al., 1987a, *J Biol Chem* 262: 13979-84; Ho et al., 1987b, *J Biol Chem* 262: 13973-8; and U.S. Patent No. 4,957,735 to Huang et al., each of which is incorporated herein by reference in

its entirety). Alternatively, the route of administration, as discussed below, can be used to target a specific cell or tissue. For example, intravenous delivery of cholesterol-containing cationic liposomes has been shown to preferentially target pulmonary tissues (Liu et al., *Nature Biotechnology* 15:167, 1997), and effectively mediate transfer and expression of genes *in vivo*. Additionally, a recombinant nucleic acid molecule can be selectively (i.e., preferentially, substantially exclusively) expressed in a target cell by selecting a transcription control sequence, and preferably, a promoter, which is selectively induced in the target cell and remains substantially inactive in non-target cells.

A liposome pharmaceutically acceptable carrier is preferably capable of remaining stable in a mammal for a sufficient amount of time to deliver a nucleic acid molecule of the present invention to a preferred site in the mammal. A liposome pharmaceutically acceptable carrier of the present invention is preferably stable in the mammal into which it has been administered for at least about 30 minutes, more preferably for at least about 1 hour and even more preferably for at least about 24 hours. A preferred liposome pharmaceutically acceptable carrier of the present invention is between about 100 and 500 nanometers (nm), more preferably between about 150 and 450 nm and even more preferably between about 200 and 400 nm in diameter.

Suitable liposomes for use with the present invention include any liposome. Preferred liposomes of the present invention include those liposomes commonly used in, for example, gene delivery methods known to those of skill in the art. Preferred liposome pharmaceutically acceptable carriers comprise multilamellar vesicle (MLV) lipids and extruded lipids. Methods for preparation of MLV's are well known in the art. According to the present invention, "extruded lipids" are lipids which are prepared similarly to MLV lipids, but which are subsequently extruded through filters of decreasing size, as described in Templeton et al., 1997, *Nature Biotech.*, 15:647-652, which is incorporated herein by reference in its entirety. Small unilamellar vesicle (SUV) lipids can also be used in the composition and method of the present invention. In one embodiment, liposome pharmaceutically acceptable carriers comprise liposomes having a polycationic lipid

composition (i.e., cationic liposomes) and/or liposomes having a cholesterol backbone conjugated to polyethylene glycol.

Preferably, the transfection efficiency of a nucleic acid:liposome complex of the present invention is at least about 1 picogram (pg) of protein expressed per milligram (mg) of total tissue protein per microgram (μg) of nucleic acid delivered. More preferably, the transfection efficiency of a nucleic acid:liposome complex of the present invention is at least about 10 pg of protein expressed per mg of total tissue protein per μg of nucleic acid delivered; and even more preferably, at least about 50 pg of protein expressed per mg of total tissue protein per μg of nucleic acid delivered; and most preferably, at least about 100 pg of protein expressed per mg of total tissue protein per μg of nucleic acid delivered.

In another embodiment of the present invention, pharmaceutically acceptable carrier can include a pharmaceutically acceptable excipient. As used herein, a pharmaceutically acceptable excipient refers to any substance suitable for delivering a therapeutic composition useful in the method of the present invention to a suitable *in vivo* site. In some embodiments, a recombinant viral vector can be administered directly to a mammal in the presence of a pharmaceutically acceptable excipient and in the absence of any additional carriers. Suitable excipients of the present invention include excipients or formularies that assist with the transport of cells and/or recombinant molecules to a site. Examples of pharmaceutically acceptable excipients include, but are not limited to water, phosphate buffered saline, Ringer's solution, dextrose solution, serum-containing solutions, Hank's solution, other aqueous physiologically balanced solutions, oils, esters and glycols. Aqueous carriers can contain suitable auxiliary substances required to approximate the physiological conditions of the recipient, for example, by enhancing chemical stability and isotonicity. Suitable auxiliary substances include, for example, sodium acetate, sodium chloride, sodium lactate, potassium chloride, calcium chloride, and other substances used to produce phosphate buffer, Tris buffer, and bicarbonate buffer. Auxiliary substances can also include preservatives, such as thimerosal, — or o-cresol, formalin and benzol alcohol.

The pharmaceutically acceptable carrier may be administered by a number of methods known in the art. In one embodiment of the invention, a pharmaceutically acceptable carrier

comprising a recombinant viral vector of the present invention is administered in an effective amount to a mammal sufficient to prevent a T-lymphocyte-mediated transplant rejection or disease recurrence. The therapeutic or pharmaceutical composition of the invention may be administered in a variety of ways, including by *in vivo* or *ex vivo* delivery methods, as described below.

Ex vivo refers to performing part of the regulatory step outside of the mammal, such as by transfecting a population of cells removed from a mammal with a recombinant viral vector of the present invention under conditions such that the protein(s) encoded by the vector is subsequently expressed by the transfected cell, and returning the transfected cells to the mammal. As used herein, the term "transfection" can be used in a broad sense to refer to any method of introducing a molecule into a cell. Methods to achieve transfection include, but are not limited to, standard transfection, electroporation, microinjection, lipofection, adsorption, viral infection (e.g., transduction), naked DNA injection, protoplast fusion, and transformation. *Ex vivo* methods are particularly suitable when the pharmaceutically acceptable carrier is a cell, and most particularly, when the cell is part of a graft, such as a tissue or whole organ.

A pharmaceutically acceptable carrier is administered to a mammal in a fashion to enable expression of the apoptosis-inducing protein as a biologically active protein in the mammal receiving a transplant. A pharmaceutically acceptable carrier can be administered to an animal in a variety of methods including, but not limited to, local administration of the composition into a site in an animal (e.g., injection locally within the area of a transplant); *ex vivo* administration (e.g., transfecting the graft cells which are to be transplanted, and/or transfecting other non-graft cells to be transplanted with the graft), peripheral administration, and systemic administration.

One method of local administration is by direct injection. Direct injection techniques are particularly useful for suppressing graft rejection by, for example, injecting the composition into the transplanted tissue. Preferably, a recombinant viral vector of the present invention alone, or contained within a pharmaceutically acceptable carrier is administered by direct injection into or locally within the area of a transplanted tissue.

Administration of a composition locally within the area of a transplanted tissue refers to injecting the composition centimeters and preferably, millimeters within the transplanted tissue. A preferred transplanted tissue to inject includes discrete organs, including, but not limited to kidney, lung, liver, and pancreas.

Preferred methods of *in vivo* administration include, but are not limited to, intravenous administration, intraperitoneal administration, intramuscular administration, intracoronary administration, intraarterial administration (e.g., into a carotid artery), subcutaneous administration, transdermal delivery, intratracheal administration, subcutaneous administration, intraarticular administration, intraventricular administration, inhalation (e.g., aerosol), intracerebral, nasal, oral, pulmonary administration, impregnation of a catheter, and direct injection into a tissue. Intravenous, intraperitoneal, and intramuscular administrations can be performed using methods standard in the art. Aerosol delivery can also be performed using methods standard in the art (see, for example, Stribling et al., *Proc. Natl. Acad. Sci. USA* 189:11277-11281, 1992, which is incorporated herein by reference in its entirety). Oral delivery can be performed by complexing a therapeutic composition of the present invention to a carrier capable of withstanding degradation by digestive enzymes in the gut of an animal. Examples of such carriers, include plastic capsules or tablets, such as those known in the art. Topical delivery can be performed by mixing a therapeutic composition of the present invention with a lipophilic reagent (e.g., DMSO) that is capable of passing into the skin.

Various methods of administration of a recombinant viral vector disclosed herein have been shown to be effective for delivery of a nucleic acid molecule to a target cell, whereby the nucleic acid molecule transfected the cell and was expressed. In many studies, successful delivery and expression of a heterologous gene was achieved using preferred pharmaceutically acceptable carriers and routes of administration of the present invention. All of the publications discussed below and elsewhere herein with regard to gene delivery and pharmaceutically acceptable carriers are incorporated herein by reference in their entirety. For example, using liposome delivery, U.S. Patent No. 5,705,151, issued January 6, 1998, to Dow et al. demonstrated the successful *in vivo* intravenous delivery of a nucleic

acid molecule encoding a superantigen and a nucleic acid molecule encoding a cytokine in a cationic liposome pharmaceutically acceptable carrier, whereby the encoded proteins were expressed in tissues of the animal, and particularly in pulmonary tissues. Liu et al., 1997, *ibid.* demonstrated that intravenous delivery of cholesterol-containing cationic liposomes containing genes preferentially targets pulmonary tissues and effectively mediates transfer and expression of the genes *in vivo*. Several publications by Dzau and collaborators demonstrate the successful *in vivo* delivery and expression of a gene into cells of the heart, including cardiac myocytes and fibroblasts and vascular smooth muscle cells using both naked DNA and Hemagglutinating virus of Japan-liposome delivery, administered by both incubation within the pericardium and infusion into a coronary artery (intracoronary delivery) (See, for example, Aoki et al., 1997, *J. Mol. Cell, Cardiol.* 29:949-959; Kaneda et al., 1997, *Ann N.Y. Acad. Sci.* 811:299-308; and von der Leyen et al., 1995, *Proc Natl Acad Sci USA* 92:1137-1141).

Delivery of numerous nucleic acid sequences has been accomplished by administration of viral vectors encoding the nucleic acid sequences. Using such vectors, successful delivery and expression has been achieved using *ex vivo* delivery (See, of many examples, retroviral vector; Blaese et al., 1995, *Science* 270:475-480; Bordignon et al., 1995, *Science* 270:470-475), nasal administration (CFTR-adenovirus-associated vector), intracoronary administration (adenoviral vector and Hemagglutinating virus of Japan, see above), intravenous administration (adeno-associated viral vector; Koeberl et al., 1997, *Proc Natl Acad Sci USA* 94:1426-1431). A publication by Maurice et al., 1999, *ibid.* demonstrated that an adenoviral vector encoding a β2-adrenergic receptor, administered by intracoronary delivery, resulted in diffuse multichamber myocardial expression of the gene *in vivo*, and subsequent significant increases in hemodynamic function and other improved physiological parameters. Levine et al. describe *in vitro*, *ex vivo* and *in vivo* delivery and expression of a gene to human adipocytes and rabbit adipocytes using an adenoviral vector and direct injection of the constructs into adipose tissue (Levine et al., 1998, *J. Nutr. Sci. Vitaminol.* 44:569-572).

In the area of neuronal gene delivery, multiple successful *in vivo* gene transfers have been reported. Millecamps et al. reported the targeting of adenoviral vectors to neurons using neuron restrictive enhancer elements placed upstream of the promoter for the transgene (phosphoglycerate promoter). Such vectors were administered to mice and rats intramuscularly and intracerebrally, respectively, resulting in successful neuronal-specific transfection and expression of the transgene *in vivo* (Millecamps et al., 1999, *Nat. Biotechnol.* 17:865-869). Bennett et al. reported the use of adeno-associated viral vector to deliver and express a gene by subretinal injection in the neural retina *in vivo* for greater than 1 year (Bennett, 1999, *ibid.*).

Gene delivery to synovial lining cells and articular joints has had similar successes. Oligino and colleagues report the use of a herpes simplex viral vector which is deficient for the immediate early genes, ICP4, 22 and 27, to deliver and express two different receptors in synovial lining cells *in vivo* (Oligino et al., 1999, *Gene Ther.* 6:1713-1720). The herpes vectors were administered by intraarticular injection. Kuboki et al. used adenoviral vector-mediated gene transfer and intraarticular injection to successfully and specifically express a gene in the temporomandibular joints of guinea pigs *in vivo* (Kuboki et al., 1999, *Arch. Oral. Biol.* 44:701-709). Apparailly and colleagues systemically administered adenoviral vectors encoding IL-10 to mice and demonstrated successful expression of the gene product and profound therapeutic effects in the treatment of experimentally induced arthritis (Apparailly et al., 1998, *J. Immunol.* 160:5213-5220). In another study, murine leukemia virus-based retroviral vector was used to deliver (by intraarticular injection) and express a human growth hormone gene both *ex vivo* and *in vivo* (Ghivizzani et al., 1997, *Gene Ther.* 4:977-982). This study showed that expression by *in vivo* gene transfer was at least equivalent to that of the *ex vivo* gene transfer. As discussed above, Sawchuk et al. has reported successful *in vivo* adenoviral vector delivery of a gene by intraarticular injection, and prolonged expression of the gene in the synovium by pretreatment of the joint with anti-T cell receptor monoclonal antibody (Sawchuk et al., 1996, *ibid.* Finally, it is noted that *ex vivo* gene transfer of human interleukin-1 receptor antagonist using a retrovirus has produced high level intraarticular expression and therapeutic efficacy in treatment of arthritis, and is

now entering FDA approved human gene therapy trials (Evans and Robbins, 1996, *Curr. Opin. Rheumatol.* 8:230-234). Therefore, the state of the art in gene therapy has led the FDA to consider human gene therapy an appropriate strategy for the treatment of at least arthritis.

Therapeutic compositions of the present invention can be administered to any animal, preferably to mammals, and more preferably to humans.

Example 1 describes the transplantation of rat islet cells into the renal subcapsular space of diabetic PVG rats. Pumps dispensing saline (controls) or purified Fas ligand (experimentals) are implanted in proximity to the graft site.

As an alternative to administration of an apoptosis-inducing protein to the graft site via the recombinant viral vector disclosed herein, transplant tissue can be grown in transgenic animals which have been genetically altered to contain the Fas ligand gene sequence. Such transgenic animals can be made by standard transgenic techniques (Example 2). Example 3 describes transplantation of islet cells from transgenic rats wherein the transplanted tissue itself is an endogenous source of Fas ligand.

In one embodiment, pharmaceutically acceptable carrier of the present invention is administered by injection or by continuous infusion from an implanted pump. Other appropriate administration forms are envisioned. For example, semipermeable implantable membrane devices that are useful as means for delivering drugs or medications are known. The encapsulation of cells that secrete neurotransmitter factors, and the implantation of such devices into the brain of patients suffering from Parkinson's disease has been described (See for example, U. S. Patent No. 4,892,538; U. S. Patent No. 5,011,472; U. S. Patent No. 5,106,627).

An effective administration protocol (i.e., administering a therapeutic composition in an effective manner) comprises suitable dose parameters and modes of administration that result in suppression of graft rejection and/or treatment of a disease. Effective dose parameters and modes of administration can be determined using methods standard in the art for a particular transplant or disease. Such methods include, for example, determination of survival rates, side effects (i.e., toxicity) and survival of graft/progression or regression of disease. In particular, the effectiveness of dose parameters and modes of administration

of a therapeutic composition of the present invention when treating a T-lymphocyte-mediated disease (discussed below) can be determined by assessing response rates. Such response rates refer to the percentage of treated patients in a population of patients that respond with either partial or complete remission of the disease. The effectiveness of dose parameters and modes of administration of a therapeutic composition of the present invention when suppressing or preventing graft/transplant rejection can be determined by assessing the survival of the graft over time.

In accordance with the present invention, a suitable single dose size is a dose that is capable of suppressing or preventing graft rejection when administered one or more times over a suitable time period. Doses can vary depending upon the disease being treated.

A suitable single dose of a recombinant viral vector encoding an apoptosis-inducing protein in a pharmaceutically acceptable carrier to administer to an animal to suppress or prevent graft rejection, is an amount capable of reducing, and preferably eliminating, destruction of the graft following transfection of the recombinant molecules into cells at or near the graft site. A preferred single dose of a therapeutic composition to treat graft rejection is from about 100 μg to about 2 milligrams (mg) of total recombinant molecules, more preferably from about 150 μg to about 1 mg of total recombinant molecules, and even more preferably from about 200 μg to about 800 μg of total recombinant molecules.

It will be obvious to one of skill in the art that the number of doses administered to an animal is dependent upon the response of an individual patient to the treatment, as well as other factors, such as whether the patient has a disease which will affect graft survival (e.g., an autoimmune disease). Thus, it is within the scope of the present invention that a suitable number of doses includes any number required to allow graft survival. A preferred protocol is monthly administrations of single doses (as described above) for up to about 1 year. A preferred number of doses of a therapeutic composition comprising pharmaceutically acceptable carrier comprising a recombinant viral vector in order to suppress graft rejection is from about 1 to about 10 administrations per patient, preferably from about 2 to about 8 administrations per patient, and even more preferably from about 3 to about 5 administrations per patient. Preferably, such administrations are given once every 2 weeks

until signs of graft survival are measured, then once a month until it is determined that treatment can be decreased or eliminated.

A preferred number of doses of a pharmaceutically acceptable carrier comprising a recombinant viral vector of the present invention in order to suppress graft rejection, is from about 2 to about 10 administrations patient, more preferably from about 3 to about 8 administrations per patient, and even more preferably from about 3 to about 7 administrations per patient. Preferably, such administrations are given once every 2 weeks signs of graft survival are measured, then once a month until it is determined that treatment can be decreased or eliminated.

In one embodiment of the method to suppress T-lymphocyte mediated graft rejection, the apoptosis-inducing protein is Fas ligand. One aspect of the present invention generally relates to the use of Fas ligand to suppress T-lymphocyte-mediated immune responses, including rejection of transplanted tissue. Such a method is disclosed in related U.S. Patent No. 5,759,536, which is incorporated herein by reference in its entirety. Such immune responses include those directed against autologous tissue in autoimmune conditions, as well as immune responses against autologous, allogeneic or xenogeneic tissue transplanted into patients in need of such tissues (e.g., transplants of porcine islet cells into patients who have or are at risk of developing diabetes mellitus). The present invention also relates to the use of Fas ligand to prevent T-lymphocyte-mediated disease recurrence and to treat T-lymphocyte-mediated primary disease (i.e., a T-lymphocyte mediated autoimmune disease). The methods of the invention involve providing an amount of Fas ligand effective to suppress T-lymphocyte-mediated rejection of transplanted tissue, disease recurrence, and/or primary disease.

Prior to the present invention, it was known that several immunologically privileged sites in mammals allow prolonged survival of transplanted allografts. The remarkable survival of islet allografts and xenografts transplanted into abdominal testes has been reported (Selawry et al, 1985, *Diabetes*, 34:1019-1024; Bellgrau et al., 1990, *Transplantation*, 50:654-657; Selawry et al., 1987, *Diabetes*, 36:1061-1067). For example, it has been shown that an unknown factor or factors released by testicular Sertoli cells

appears to be responsible for the protection of the intratesticular islet allografts and xenografts against rejection (Selawry et al., 1991, *Transplantation*, 52:846-850). This unknown factor(s) has been reported to inhibit the production of IL-2 *in vitro*.

In addition, Sertoli cells have been used to establish an immunologically privileged site *in vivo* in the renal subcapsular space (Selawry et al., 1993, *Cell Transplantation*, 2:123-129). Briefly, diabetic PVG rats received rat islet cell grafts with and without Sertoli cells and with and without cyclosporine (CsA). The results showed that 70%-100% of the recipient rats receiving islet cells alone, islet cells and CsA, or islet cells and Sertoli enriched cells, remained hyperglycemic. In contrast, prolonged normoglycemia in excess of 100 days was achieved in rats receiving a combination of islet cells, Sertoli enriched cells, and CsA.

The present inventors discovered for the first time that the factor released by testicular Sertoli cells which is responsible for the protection of the intratesticular islet allografts and xenografts against rejection is the Fas ligand. It was not until the discovery of this heretofore unknown mechanism of T-lymphocyte suppression in an immunopriveleged site that the novel, site-specific, and highly effective methods for suppressing T-lymphocyte mediated graft rejection, T-lymphocyte-mediated disease recurrence, and T-lymphocyte-mediated primary disease by providing exogenous Fas ligand were appreciated.

The Fas ligand, which is the naturally occurring ligand of Fas, was recently purified and identified as a 40 kD membrane glycoprotein (Suda et al., 1993, *Cell,* 75:1169-1178). The purified Fas ligand exhibits cytolytic activity against cells expressing Fas. Prior investigators, however, failed to appreciate that Fas ligand is responsible for the absence of T-lymphocyte responses in "immunopriveleged sites", and that exogenous expression of Fas ligand by transfected cells or by delivery of Fas ligand to a cell via a viral vector is a safe and effective means of suppressing T-lymphocyte mediated graft rejection and/or treating T-lymphocyte mediated primary disease and disease recurrence. Instead, prior investigators cautioned <u>against</u> therapies designed to ligate Fas on cells *in vivo*, in view of studies which showed that administration of anti-Fas antibody *in vivo* to an animal was lethal (Ogasawara et al., 1993, *Nature*, 364:806-809). Similarly, other investigators suggested that soluble Fas

ligand may contribute to human disease in a similar manner as the above-referenced anti-Fas antibody (Suda et al., 1993, *supra*).

Prior to identification of the Fas ligand, it was known that ligation of Fas on the surface of lymphocytes which were activated chronically with IL-2 *in vitro* (i.e., artificially and non-specifically activated) resulted in apoptosis of the cell expressing Fas (Owen-Schaub, 1992, *Cell. Immunol.*, 140:197-205). These studies, however, failed to show that induction of apoptosis occurred in freshly isolated lymphocytes, in cultured non-activated lymphocytes, or in early activated lymphocytes, and suggested that Fas was not the sole mediator of this apoptotic cell death. Other studies added to the confusion as to whether normally activated T cells are primed for Fas-mediated killing (Klas et al., 1993, *Int. Immunol.*, 5:625-630). It was not until the present invention that it was demonstrated that ligation of Fas by its natural ligand, the Fas ligand, *in vivo* is sufficient to cause apoptotic death in cells bearing Fas, including normally activated T cells.

Furthermore, as discussed above, the present inventors are the first to appreciate that Fas ligand, provided exogenously (e.g., delivered and/or expressed by a viral vector), is safe and effective *in vivo* for suppressing T-lymphocyte-mediated graft rejection. Prior to the present invention, many proposed therapies for suppressing graft rejection involved <u>down-regulating</u>, <u>eliminating</u> or <u>masking</u> molecules on the surface of cells in a graft to prevent recognition of the graft by T-lymphocytes directed against the graft (See, for example, Faustman et al., 1994, U.S. Patent No. 5,283,058). In contrast, the methods of the present invention involve purposefully expressing and/or upregulating the expression of a molecule (i.e., Fas ligand) on the surface of cells in the graft or in cells cotransplanted with the graft, in order that the Fas ligand will specifically interact with T-lymphocytes directed against the graft.

In activation of the immune system, T-lymphocytes (i.e., T cells) are presented with a foreign antigen. A T cell response occurs when a T cell receptor (TCR) recognizes an antigenic peptide (e.g., a foreign antigen) bound to an MHC protein, thereby altering the activity of the T cell bearing the TCR. As used herein, a "T cell response" can refer to the activation, induction of anergy, or death of a T cell that occurs in response to an interaction

between a molecule on the T cell with another molecule. As used herein, "activation" of a T cell refers to induction of signal transduction pathways in the T cell resulting in production of cellular products (e.g., interleukin-2) by that T cell. Activated T-lymphocytes further respond by differentiating into effector cells, and the effector cells then clear the foreign antigen. "Anergy" refers to the diminished reactivity by a T cell to an antigen. As used herein, "T cell death" refers to the permanent cessation of substantially all functions of the T cell. One type of T cell death is apoptosis.

In rejection of an allograft, the immune system of the recipient animal has not previously been primed to respond because the immune system for the most part is only primed by environmental antigens. In other words, tissues from other members of the same species have not been presented in the same way that, for example, viruses and bacteria have been presented. In the case of allograft rejection, immunosuppressive regimens are typically designed to prevent the immune system from reaching the effector stage. However, the immune profile of xenograft rejection may resemble disease recurrence more than allograft rejection. In the case of disease recurrence, the immune system has already been activated, as evidenced by destruction of the native islet cells. Therefore, in disease recurrence the immune system is already at the effector stage. The Fas ligand is able to suppress the immune response to both allografts and xenografts because lymphocytes activated and differentiated into effector cells express Fas, and thereby are susceptible to the Fas ligand.

According to the present invention, the terms "graft" and "transplant" can be used interchangeably. Similarly, a graft can be transplanted into a recipient, and a transplant can be grafted into a recipient.

A reaction or disease is considered to be T-lymphocyte-mediated when T-lymphocytes are required in mediating the reaction or disease effect. Where cells of the tissue for transplantation (the "donor" tissue) bear on their surfaces foreign histocompatibility antigens, these antigens cause cytotoxic T-lymphocyte activation in recipients, terminating in donor cell destruction after several sequential activation stems. The cascade is initiated by conjugate formation between the antigen-specific T-cell receptor on host T-lymphocytes and the major histocompatibility antigens on the donor cell. Conjugate formation is followed

by T-lymphocyte-mediated activation, resulting in donor cell death. This process can eventually result in rejection even in intra-species transplantation. According to the invention, this problem is addressed by suppressing the T-lymphocyte response prior to the stage where donor cell destruction is initiated.

The Fas ligand may be used to treat chronic transplant rejection. It is recognized by the art that most transplants undergo a chronic graft destructive process. The mechanism of chronic transplant rejection differs from conventional allograft immunity and conventional immunosuppression has been ineffective in its treatment. Chronic draft rejection may be treated with the Fas ligand, resulting in successful engraftment for longer periods of time and allowing donor tissue to be used for new recipients.

The Fas ligand may also be used to treat acute graft rejection. Treatment with the Fas ligand should provide a more specific treatment for activated cells, that is, for cells attacking the transplant tissue, not all the T-lymphocytes present in the immune system.

This invention addresses that need by providing methods which allow the use of non-human tissue for transplantation into a human patient in need thereof. The method of the invention prevents rejection of xenogeneic tissue. The invention thus permits not just intra-species transplantation of tissues and organs, but xenografts as well, opening up the possibility of "farming" of donor organs and tissues in non-human mammals for transplantation into human patients. In the case of xenografts, this invention may be practiced along with other methods for masking, modifying, or eliminating undesirable antigens on the surface of donor cells, such as the method described in U.S. Patent No. 5,283,058, which is incorporated herein by reference in its entirety.

Another embodiment of the present invention relates to a method of inducing apoptosis in cells of a recipient mammal. In particular, this method is useful for inducing apoptosis in a particular cell population to be eliminated, such as cancer cells at a site of a cancer (e.g., a tumor). The method comprises introducing into the mammal a pharmaceutically acceptable carrier which comprises a recombinant viral vector comprising a nucleic acid sequence encoding an apoptosis-inducing protein. In one embodiment, the pharmaceutically acceptable carrier comprises: a) a recombinant nucleic acid molecule

comprising a nucleic acid sequence encoding a protein that inhibits apoptosis; and (b) a recombinant viral vector comprising a nucleic acid sequence encoding an apoptosis-inducing protein. In this embodiment, the recombinant nucleic acid molecule of (a) is preferably contained within the recombinant viral vector, although the recombinant nucleic acid molecule can be administered as a separate molecule. When the nucleic acid sequence encoding the apoptosis-inhibiting protein is within the recombinant viral vector, the nucleic acid sequence encoding this protein and the nucleic acid sequence encoding the apoptosis-inducing protein can be operatively linked to the same or different transcription control sequences, and when linked to the same transcription control sequence, are preferably separated by an IRES.

Preferably, the recombinant viral vector is delivered to a target cell which is at or adjacent to the site of the cancer, or in one embodiment, the pharmaceutically acceptable carrier is a cell which in transfected with the recombinant viral vector and delivered to the site of the cancer. Therefore, the recombinant viral vector can be propagated in the carrier cell and released at the site of the cancer to infect cancer cells and cause apoptosis in the cancer cells, the recombinant viral vector can be expressed directly in cancer cells or cells adjacent to the cancer cells, or the recombinant viral vector can be expressed by the carrier cell and interact with cancer cells to cause apoptosis. The present inventors have found that the recombinant viral vector encoding an apoptosis-inducing protein is effective to induce apoptosis in tumor cells at the site of delivery, even though some of the tumor cells are not infected with the vector. The presence of the apoptosis-inhibiting protein in the vector may protect a carrier cell in some embodiments, and in other embodiments, such protein may attenuate the apoptotic response so that apoptosis is controlled and limited to tumor cells. This method of the present invention can be used to treat any cancer, and cancers into which the recombinant viral vector is easily introduced are particularly preferred. Such cancers include, but are not limited to, prostate cancer, lung cancer, brain cancer, lymphoma (e.g., Sezary Syndrome, mycosis fungoides, T cell lymphoma), leukemia, and metastatic cancers which reside in these tissues (e.g., a liver cancer that has metastasized to the lung).

In this embodiment of the present invention, the preferred recombinant viral vectors are derived from any of the viral vectors disclosed previously herein. In the case of lymphomas and leukemias, the recombinant viral vector is preferably from a lentivirus. In this embodiment, the vectors are preferably administered systemically. In other types of cancer, preferred routes of administration are those routes which most directly deliver the recombinant viral vector to the site of cancer with limited exposure to other tissues. For example, a recombinant viral vector of the present invention is preferably delivered to lung cancers by aerosol or intratracheal delivery, and the vector is preferably delivered to brain cancers by direct injection into the tumor.

Yet another embodiment of the present invention relates to methods for suppressing a T-lymphocyte-mediated disease in a recipient mammal. This method includes introducing (i.e., administering) into a mammal a pharmaceutically acceptable carrier comprising a recombinant viral vector comprising a nucleic acid sequence encoding a protein that induces apoptosis operatively linked to a transcription control sequence. In one embodiment of this method, the pharmaceutically acceptable carrier can additionally comprise a recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a protein that inhibits apoptosis operatively linked to a transcription control sequence. In this embodiment, the nucleic acid sequence encoding the apoptosis-inhibiting protein is preferably contained within the recombinant viral vector, although the recombinant nucleic acid molecule can be administered as a separate molecule, particularly when delivered to a cell by *ex vivo* methods. When the nucleic acid sequence encoding the apoptosis-inhibiting protein is within the recombinant viral vector, the nucleic acid sequence encoding this protein and the nucleic acid sequence encoding the apoptosis-inducing protein can be operatively linked to the same or different transcription control sequences, and when linked to the same transcription control sequence, are preferably separated by an IRES. Such vectors, recombinant molecules, apoptosis-inducing proteins and apoptosis-inhibiting proteins have been described above. When a recombinant nucleic acid molecule encoding an apoptosis-inhibiting protein is included, the method has the advantage of protecting a cell which expresses the apoptosis-inducing protein from elimination itself. In a preferred embodiment, the apoptosis-inducing

protein is Fas ligand. For example, lymphocytes activated by disease as well as by the introduction of foreign grafts express Fas, and therefore, are susceptible to treatment with Fas ligand. In general terms, the Fas ligand as an immunosuppressive agent is most active against a primed or activated immune system. The primed or activated immune system may he associated with disease conditions in which either T-lymphocytes or B-lymphocytes are activated. Activated T-lymphocytes are associated with disease in graft versus host reactions (e.g., bone marrow transplantation) and most forms of autoimmunity, including but not restricted to, multiple sclerosis, rheumatoid arthritis, lupus, and myasthenia gravis. Fas expressing leukemia may also be susceptible to treatment with the Fas ligand, since Fas is expressed by B- and T-lymphocyte tumors.

In order to treat an animal with a T-lymphocyte-mediated disease, a pharmaceutically acceptable carrier of the present invention is administered to the animal in an effective manner such that the composition is capable of treating that disease. For example, a recombinant viral vector of the present invention, when administered to an animal in an effective manner, is able to suppress effector cell immunity in a manner that is sufficient to alleviate the disease afflicting the animal. According to the present invention, treatment of a disease refers to alleviating a disease and/or preventing the development of a secondary disease (e.g., recurring disease) resulting from the occurrence of a primary disease.

Such a therapeutic composition of the present invention is particularly useful for the treatment of autoimmune diseases, including but not limited to, insulin dependent diabetes mellitus, multiple sclerosis, systemic lupus erythematosus, myasthenia gravis, rheumatoid arthritis, psoriasis, polyarteritis, immune mediated vasculitides, immune mediated glomerulonephritis, inflammatory neuropathies and sarcoidosis.

A single dose of recombinant viral vector to administer to an animal to treat an autoimmune disease is from about 0.1 μg to about 200 μg of total recombinant molecules per kilogram (kg) of body weight, more preferably from about 0.5 μg to about 150 μg of total recombinant molecules per kg of body weight, and even more preferably from about 1 μg to about 10 μg of total recombinant molecules per kg of body weight. The number of doses of the recombinant viral vector to be administered to an animal to treat an autoimmune disease

is an injection about once every 6 months, more preferably about once every 3 months, and even more preferably about once a month.

A pharmaceutically acceptable carrier of the present invention can be administered to treat an autoimmune disease by any method and dose previously disclosed herein for administration of a viral vector and/or pharmaceutically acceptable carrier of the present invention. In one embodiment, the pharmaceutically acceptable carrier is administered by local administration, preferably direct injection at the site of the autoimmune response. Although the pharmaceutically acceptable carrier and viral vector can be designed to have prolonged expression (e.g., weeks to months) at the site of treatment, prolonged expression is not required to have a therapeutic effect and in some instances, short-term expression is preferred. Other preferred routes and protocols of administration have been previously described in detail herein.

The invention may be used to treat a number of human disease conditions resulting from destruction of endogenous cells, such as the destruction of insulin producing pancreatic islet beta cells in diabetes. An important feature of the invention is that it makes possible the use of non-human mammals as tissue and organ donors for human patients. The above methods describe the use of the invention to treat diabetic human patients by transplantation of xenogeneic islet cells. The xenogeneic islet cells may be obtained for example, normal or transgenic pigs expressing the Fas ligand protein. Example 4 describes transplantation of transgenic porcine islet cells into a diabetic human patient.

Transgenic animals

Preferably, a transgenic non-human animal of the present invention is a mammal including, but not limited to, farmed mammals, primates and rodents. In one embodiment, a preferred transgenic non-human animal of the present invention is a rodent, and even more preferably, a rat or a mouse. In another embodiment, a preferred transgenic non-human animal is a mammal which can be used to provide donor organs and/or tissues to a human patient, including, but not limited to, primates and pigs.

According to the present invention, a transgenic non-human animal is a non-human animal which includes a recombinant nucleic acid molecule (i.e., transgene) that has been

introduced into the genome of the non-human animal at the embryonic stage of the non-human animal's development. As such, the transgene will be present in all of the germ cells and somatic cells of the non-human animal. Methods for the introduction of a transgene into a mouse embryo, for example, are known in the art and are described in detail in Hogan et al., "Manipulating the Mouse Embryo. A Laboratory Manual", Cold Spring Harbor press, Cold Spring Harbor, NY, 1986, which is incorporated by reference herein in its entirety. For example, a recombinant nucleic acid molecule (i.e., transgene) can be injected into the male pronucleus of a fertilized mouse egg to cause one or more copies of the recombinant nucleic acid molecule to be retained in the cells of the developing mouse. A mouse retaining the transgene, also called a "founder" mouse, usually transmits the transgene through the germ line to the next generation of mice, establishing transgenic lines. According to the present invention, a transgenic mouse also includes all progeny of a transgenic mouse that inherit the transgene.

Transgene sequences can be cloned using a standard prokaryotic cloning system, and the transgene products can excised from the prokaryotic vector, purified, and injected into the pronuclei of fertilized eggs from the desired transgenic animal. Stable integration of the transgene into the genome of the transgenic embryos allows permanent transgenic animal lines to be established.

The method of the invention may also be used to prevent a recurring disease which resulted in destruction of endogenous tissue. For example, disease recurrence mediated by T-lymphocytes directed to islet β cell antigens results in destruction of grafted islet cells. Therefore, providing Fas ligand to the graft site prevents recurrence of diabetes and allows normoglycemia to be achieved in recipient mammals by suppressing the immune response directed to islet β cell antigens.

The following examples are provided for the purposes of illustration and are not intended to limit the scope of the present invention.

EXAMPLES

Example 1

The following examples demonstrates suppression of T-lymphocyte-mediated rejection of transplanted tissue by administration of Fas ligand.

Islet preparation. Freshly isolated islet cells from a rat are prepared according to .known methods. See, for example, London et al. (1990) Transplantation 49:11091113. Under appropriate circumstances, islet cells may be pretreated prior to transplantation to conceal (,'mask") donor antigens or modify graft immunogenicity by methods known in the art, for example, those described in U. S. Patent No. 5,283,058,

Purified Fas ligand. Purified Fas ligand may be obtained from a mammalian source or produced *in vitro* as a recombinant protein. In one embodiment of the invention, purified Fas ligand is obtained from a naturally occurring source. A simple method for large scale purification of Fas ligand from cultured cells has been reported (Suda & Nagata (1994) supra) . Briefly, cells expressing Fas ligand are cultured and harvested. A solubilized membrane fraction is purified by affinity purification, and the Fas ligand eluted as described by Suda & Nagata (1994), supra.

In another embodiment, Fas ligand is produced by recombinant DNA methods, utilizing the genes coding for Fas ligand. Expression of a recombinant rat Fas ligand has been obtained (Suda et al. (1993) Cell 75:IIG9-1178). The amino acid sequences of many proteins are highly conserved across a variety of mammalian species. As a consequence of the conservation of the nucleotide sequences there is considerable conservation of the nucleotide sequences of the genes that encode these proteins. Therefore, it is generally true that the gene encoding the Fas ligand in one mammalian species can cross-hybridize (i.e. form a stable double-stranded DNA hybrid) with the genes encoding that factor in other mammalian species under appropriate annealing conditions. This property may he used to identify cloned human DNA segments that include the gene for Fas ligand. For example, the human gene encoding the Fas ligand may be identified by screening a human genomic library using a ^{32}P-labeled probe derived from the rat cDNA sequence of the Fas ligand (Suda et al. (1993) supra). Suitable host cells transformed with a vector containing DNA encoding the

human Fas ligand are cultured under conditions for amplification of the vector and expression of the Fas ligand, and Fas ligand is harvested.

Bioassay of Fas ligand. The biological activity of purified Fas ligand is assessed *in vitro* with the, cytotoxicity assay described by Suda & Nagata (1994) supra.

Transplantation of rats and administration of Purified Fas ligand . Diabetic PVG rats are grafted with islet cells and implanted with pumps dispensing saline (controls) or purified Fas ligand (experimental) as follows. Diabetic PVG rats are anesthetized with methoxyflurane USP and the left flank opened to expose the kidney. Islets cells (10 islets/g of body weight) are injected under a renal capsule as described by Selawry & Cameron (1993) supra. A pump programmed to dispense either saline or purified Fas ligand over an empirically-determined period of time is implanted under the renal capsule. Cyclosporine (CsA) may be injected subcutaneously 25 mg/kg per day for a seven day period.

Recipient rats are evaluated for plasma glucose levels. Urine volumes and urine glucose contents are obtained and determined as described (Selawry & Cameron (1993) supra). Recipient rats receiving Fas ligand become normoglycemic over a prolonged period of time.

Example 2

The following example demonstrates production of transgenic mammals containing DNA encoding the Fas ligand.

A transgenic rat whose germ cells and somatic cells contain the Fas ligand gene is produced by methods known in the art. See, for example, U. S. Patent No. 4,736,866 describing production of a transgenic mammal, herein incorporated by reference. Generally, the DNA sequence encoding the Fas ligand is introduced into the animal, or an ancestor of the animal, at an embryonic stage (preferably at the one-cell, or fertilized oocyte, stage, and generally not later than about the 8-cell stage). There are several methods known to the art of introducing a foreign gene into an animal embryo to achieve stable expression of the foreign gene. One method is to transfect the embryo with the gene as it occurs naturally, and select transgenic animals in which the foreign gene has integrated into the chromosome at a locus which results in its expression. Other methods involve modifying the foreign gene

or its control sequences prior to introduction into the embryo. For example, the Fas ligand gene may be modified with an enhanced, inducible, or tissue-specific promoter.

Tissues of transgenic rats are analyzed for the presence of Fas ligand, either by directly analyzing RNA, by assaying the tissue for Fas ligand, or by assaying conditioned medium for the secreted Fas ligand. For example, cells obtained from the transgenic rat are cultured in the presence of ^{35}S-methionine, the supernatant subjected to immunoprecipitation with antibodies to Fas ligand. Precipitated proteins are resolved by reducing SDS - polyacrylamide gel electrophoresis, and visualized by autoradiography. Conditioned medium may also be tested for *in vitro* cytotoxic activity by the method of Suda & Nagata (1994), *supra*, or by performing chromium release assays as described in Example 10.

Example 3

The following example shows transplantation of transgenic islet cells expressing the Fas ligand.

Islet cells are obtained from the transgenic rat of Example 2 and grafted into diabetic PVG rats by the methods described in Example 1. Recipient rats, evaluated as described above, achieve normoglycemia for prolonged periods of time.

Example 4

The following example demonstrates the transplantation of transgenic porcine islet cells into a human diabetic patient.

A transgenic pig is obtained all of whose germ cells and somatic cells contain a recombinant DNA sequence encoding human Fas ligand. The human Fas ligand DNA sequence was introduced into the pig by methods known to the art. Islet cells are obtained from the transgenic pig by the methods described in Example 2 and are grafted into diabetic human patient by methods known in the art. The human patient, evaluated appropriately, achieves normoglycemia for prolonged periods of time.

Example 5

The following example demonstrates production of Fas ligand mRNA by isolated Sertoli cells.

cDNA synthesis. Total RNA from purified rat Sertoli cells was isolated from cell pellets by the method of Chomczynski and Sacchi (1987) Anal. Biochem. 162:156. The RNA (5 μg) was first denatured in methyl mercuric hydroxide (10 mM final concentration) (Alfa Products, Ward Hill, MA) and converted to cDNA in Taq (*Thermus aquaticus*) DNA polymerase buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$ and 0.01% gelatin) in the presence of RNA guard (20 units) (Pharmacia, Piscataway, NJ), β-mercaptoethanol (40 mM), dNTPs (0.5 mM) (Pharmacia), 1 μg random hexamers (Pharmacia) and AMV (Avian myeloblastosis virus) reverse transcriptase (20 units) (Life Sciences Inc., St. Petersburg, FL) in a 50 μl reaction for 90 min at 42°C.

PCR amplification. Following synthesis, 5 μl of the cDNA was transferred to a tube on ice containing 200 mM dNTPs (Pharmacia), Taq polymerase buffer containing 1.5 mM $MgCl_2$, Taq DNA polymerase (1 unit) (Perkin Elmer Cetus, Norwalk, CT) and the rat Fas ligand specific oligonucleotide primers 5′-GCCCGTGAATTACCCATGTC-3′ (SEQ ID NO:1) and 5′-TGGTCAGCAACGGTAAGATT-3′ (SEQ ID NO:2) (forward and reverse, respectively). The samples were overlaid with light mineral oil (Sigma Chemical Corp., St. Louis, MO) and transferred to a thermal cycler (MJ Research, Inc., Watertown, MA). Following heating to 94°C for 5 min to denature DNA/RNA complexes, the samples were amplified for 28 cycles of 1 min at 94°C, 1.5 min at 55°C, and 2 min at 72°C, followed by a final 10 min extension at 72°C.

Detection. 20 μl of the starting 50 μl reaction was separated by electrophoresis through a 1.6% agarose gel. The following samples were run: mRNA from Sertoli cells incubated at 32°C (lane 1) or at 37°C (lane 3) ; mRNA from Sertoli cells from a second animal incubated at 32°C (lane 2) or at 37°C (lane 4). The DNA in the gel was then transferred to nitrocellulose filters (Schleicher & Schuell, Keene, NH) according to the method of Southern (1975) J. Mol. Biol. 98:503. The filters were UV cross linked (Stratagene, San Diego, CA) and hybridized at 37°C overnight in a solution containing 6X SSC (1X SSC = 0.15 M sodium chloride and 0.015 M sodium citrate), 1X Denhardts (0.02% each Ficoll 400, bovine serum albumin, and polyvinylpyrrolidone), 20 μg/ml wheat germ tRNA, 0.1% SDS and 0.05% sodium pyrophosphate plus the ^{32}p end-labeled Fas ligand

specific oligonucleotide 5′-AACATAGAGCTGTGGCACC-3′ (SEQ ID NO:3). After extensive washing in 6X SSC plus 0.05% sodium pyrophosphate at 47°C, the filters were dried and exposed to Kodak X-Omat film.

An autoradiograph of the amplified rat Sertoli cell mRNA was obtained. Lanes 1 and 3 are mRNA from cells incubated at 32°C or 37°C, respectively; lanes 2 and 4 are mRNA from Sertoli cells taken from a second animal cultured at 32°C or 37°C, respectively. These results show that Sertoli cells are the dominant, if not exclusive, source of Fas ligand in the testis.

<u>DNA sequencing</u>. The PCR product was determined to be identical to that published by Suda et al. (1993) Cell 75:1169 by standard DNA sequencing methodology of Sanger et al. (1977) Proc. Natl. Acad. Sci. USA 78:5453.

<u>Example 6</u>

The following example demonstrates the effect of Fas ligand on grafted testicular survival.

To test whether the absence of a functional Fas ligand molecule prohibited Sertoli cells from providing their immunosuppressive function, the present inventors transplanted testicular tissue from male B6-Gld or genetically compatible but Fas ligand operative C57BL/6 (B6) mice into BALB/c recipients. The B6-Gld and B6 strains are identical at the major histocompatibility complex (MHC) and also share essentially all other (minor) histocompatibility antigens with the exception of the Fas ligand. B6-Gld carries a point mutation in the Fas ligand gene (Takahashi et al. (1994) Cell 7:969). B6-Gld and B6 differ from the BALB/c strain at both the MHC and multiple minor loci.

Testicular drafts of B6-Gld or B6 tissue were transplanted under the kidney capsule of BALB/c recipients. A mouse is weighed and injected with Avertin (12 ml/g), and anesthetized with Metophane. The mouse is shaved under the rib cage on the left side, and a small incision (about 8 mm) is made through the body wall. The adipose tissue attached to the posterior end of the kidney is pulled such that the kidney is externalized. The kidney is kept moist with Hank's Basic Salt Solution (HBSS). A straight incision is made on the posterior end of the kidney and the kidney capsule carefully loosened from the kidney with

a probe. The tissue to be transplanted is deposited under the capsule and gently moved to the anterior part of the kidney with a probe. The kidney is replaced inside the body and the incision closed.

<u>Blood Clot Transplantation Procedure</u>. This procedure is designed to permit groups of cells or non-clustered islets to be transplanted under the recipient host kidney capsule. Embedding the cells within the clot allows the transplanter to place them in a relatively defined position from which they will not move.

Cells to be transplanted are transferred to a siliconized 15 ml centrifuge tube and allowed to settle for 5 min. Cells in a cell suspension are transferred to a siliconized 15 ml centrifuge tube and centrifuged at 300 x g for 5 min. Most of the supernatant is removed and the cells resuspended in the remaining 200 μl medium. The cells are resuspended and transferred to a 300 μl microfuge tube, centrifuged at 500 - 1.000 x g for 30 sec and placed on ice. Most of the supernatant is removed, leaving about 4-5 mm fluid. With a scalpel, the top is cut just above the fluid level, and the remaining fluid removed with a capillary tube. Blood is drawn from the tail vein of the recipient animal and approximately 5 μl blood added to the cell pellet. A clot is allowed to form for 10 min. Residual sera is drawn off. The cells are embedded within the clot matrix and are not easily dislodged. The cell clot may then be transplanted into the kidney capsule.

<u>Results</u>. On days 2 and 7 (B6-Gld) or days 2, 7, and 28 (B6), the grafted tissue was analyzed macroscopically and microscopically for graft rejection. A recipient BALB/c mouse was euthanized with an overdose of penthrane. The kidney containing the graft was removed, fixed in a formal saline buffer solution and processed by routine histologic techniques. The kidney was embedded in paraffin after which 5 μl sections were cut and stained with hematoxylin and eosin. Renal tissue obtained from BALB/c kidney engrafted with B6 tissue appeared structurally normal by light microscopy. Transplanted tissue was observed adjacent to the kidney capsule. It appeared no different in morphology from that which was observed when histocompatible-genetically identical BALB/c tissue was used as the source of donor tissue. In B6-Gld engrafted kidney there was extensive infiltration of lymphocytes in the graft by day 2, and the architecture of the testis tissue was disrupted. The

renal tissue also showed obvious lymphocytic infiltration adjacent to as well as within the craft. By day 7, there was little recognizable testis tissue and infiltrate was diminished, indicating that the destructive process had peaked before this time. These findings establish the role of the Fas ligand in immunosuppression, and show that the absence of a functional Fas ligand gene protects transplanted testicular tissue from graft rejection.

Example 7

The following example demonstrates the effect of Fas ligand on Sertoli cell immunosuppressive activity.

To establish if isolated Sertoli cells could duplicate the results obtained with testis tissue grafts (Example 6), Sertoli cells were isolated and purified from testicular tissue of B6-Gld and B6 mice and transplanted as single cell suspensions under the kidney capsule in BALB/c mice, essentially as described by Selawry and Cameron (1993) Cell Transplantation 2:123 and Example 6 above. Testis were removed from mice and cut into small pieces in 5 ml HAM'S F12/DMEM media (Ham's media). The tissue was placed in a 50 ml tube, 25 ml Ham's media added, and pelleted by centrifugation at 800 x g for 2-5 min. The pellet was resuspended in 20 ml Ham's media containing 20 mg trypsin and 0.4 mg DNAse. The resulting mixture was placed in a 250 ml flask in a shaking water bath at 37°C for 30 min, and pelleted at 800 x g for 2-5 min. The cell pellet was resuspended at room temperature for 10 min in 20 ml of a solution containing 1 M glycine, 2 mM EDTA, 0.01% soy bean trypsin inhibitor, and 0.4 mg DNAse. The mixture was centrifuged as above, and the cell pellet washed twice. Cells were resuspended in 20 ml Ham's media containing 10 mg collagenase, and placed in a shaking water bath at 37°C for 5 minutes, pelleted, and resuspended in 20 ml Ham's media containing 20 mg collagenase and 0.1 mg DNAse. The sample was transferred to a 250 ml flask placed in a rocking water bath at 37°C for 30 min. The cells were pelleted and washed as described above. Cells were resuspended in 10 ml Ham's media containing 20 mg hyaluronidase and 0.1 mg DNAse, and placed in 250 ml flask in a rocking water bath at 37°C for 30 min. Cells were pelleted and washed. The final pellet was kept on ice until transplanted under the kidney capsule. The pellet may be clotted with blood drawn from the host mouse (see blood clot transplantation procedure described above).

Results identical to those described in Example 6 were obtained. B6-Gld Sertoli cells transplanted under the kidney capsule of histoincompatible BALB/c recipient mice remained intact. These results establish that the Fas ligand is an effective immunosuppressive factor responsible for the immunosuppressive effects of Sertoli cells.

Example 8

The following example demonstrates the diagnostic use of Fas ligand expression for selecting donor tissue or recipient transplantation site.

The discovery of the relationship between a functioning Fas ligand gene and protection from graft rejection may be applied diagnostically. The ability of various non-lymphoid tissue sources to express Fas ligand, detected either by examination of tissue with monoclonal antibodies to Fas ligand or by assessing Fas ligand mRNA by RT-PCR, allows prediction of the capacity for a specific tissue to be retained or rejected following transplantation. Tissues expressing a high level of Fas ligand provide a preferred site for successful organ engraftment. Screening donor tissue for Fas ligand expression will also aid in predicting transplantation success.

Example 9

The following example demonstrates that prostate cancer (PC) cell lines show enhanced sensitivity to hFasL versus agonistic antibody.

Nontransfected cell cultures: The human PC cell lines, LNCaP, ALVA-31, TSU-Pr1, JCA-1, PPC-1, PC-3 and DU 145 were obtained and maintained as described previously (Hedlund et al., 1998, *Prostate* 36:92-101). K562, a human erythroid leukemia cell line was purchased from the American Type Culture Collection (Rockville, MD, USA) and were maintained in RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 7.5% heat-inactivated FBS (Hyclone, Logan UT, USA) and 2 mM L-glutamine (Gibco) at 37°C in 95% air and 5% CO_2.

Stably transfected cell lines: (1) K582-hFasL and K562-neo: human K562 chronic myelogenous leukemia cells were transfected with plasmids encoding human FasL and neomycin phosphotransferase (K562-hFasL) or with a plasmid encoding neomycin phosphotransferase alone (K562-neo) as described elsewhere. (2) L1210-Fas: this

transfected mouse lymphocytic leukemia cell line expresses high levels of mouse Fas and was kindly provided by Dr. P. Golstein (Marseilles, France). All transfected cell lines were routinely cultured in the presence of 600 μg/ml active Geneticin, using the medium and conditions described for nontransfected cells.

In this experiment, prostate cancer cells were labeled with ^{51}Cr and were co-incubated for 18 h at various effector-to-target ratios with either K562-hFasL or K582-neo. Each of seven PC targets listed in Table 1 below was tested simultaneously in cytotoxicity assays to allow for direct comparison of their apoptotic potentials. L1210-Fas cells served as positive controls since they are highly sensitive to Fas crosslinking. As shown in Fig. 1A, K562-hFasL induced lysis of L1210-Fas in a dose dependent fashion, whereas no specific lysis was detected when K562 cells transfected with neomycin phosphotransferase alone were used as effectors. When used as a target, the PC cell line ALVA-31 behaved similarly (Fig. 1B), with cell lysis reaching 63% at an effector to target (E:T) ratio of 10:1. A summary of the results with all eight target cell lines is presented in Table 1. The cell lines are listed in order of descending apoptotic potentials as observed in the cytotoxicity assay. Also presented in Table 1, are the results from previous studies using an agonistic mouse IgM anti-hFas antibody. These results demonstrate a markedly enhanced activity of membrane-expressed hFasL *versus* agonistic anti-Fas antibody.

TABLE 1

Specific lysis of human prostate cancer cell lines by K562-FasL transfectants at a 10:1 effector to target ratio. A comparison with cell lysis achieved using an anti-Fas IgM antibody in previous studies.

Target Cell line	% Lysis by K562-FasL (±S.D.)	% Lysis by anti-Fas IgM antibody
L1210-Fas	60 ± 10	(not determined)
ALVA-31	63 ± 5	28%
TSU-Pr1	52 ± 13	undetectable
PPC-1	48 ± 1	10%
JCA-1*	47 ± 6	10%
LNCaP	27 ± 3	undetectable
DU 145*	21 ± 2	undetectable
PC-3	19 ± 5	undetectable

* JCA-1 and DU 145 showed slight but statistically significant lysis by the vector-transfected K562 clone (14±1, and 4±0.2%, respectively)

An interesting feature of the prostate is that it is believed to escape immune surveillance to some extent because it lacks afferent lymphatics and because of the immunosuppressive properties of seminal fluid. In fact, the normal prostatic epithelium has been found to co-express Fas and FasL, as indicated by studies with both mouse and human prostatic tissue. Therefore, the present inventors considered that it may be more relevant to test the sensitivities of the PC cell lines to internally expressed FasL through transgene methods of gene expression, rather than to FasL-expressing effector cells. Given that FasL expression was expected to induce apoptosis, the present inventors chose to develop an adenoviral system of gene transduction rather than to establish stably transfected cell lines or to rely on the limited efficiency of transient transfection methods. Adenovirus entry has been shown to be highly efficient in cells that express the integrin family of adhesion molecules and this is a common feature of many PC cell lines.

Example 10

The following example shows the construction of recombinant adenovirus encoding FasL and its propagation in 293-crmA cells.

cDNA encoding mFasL was inserted into the E1 region of a replication deficient human adenovirus 5 construct under the control of the CMV immediate early promoter to produce Ad5*dl*327CMV-mFasL (Ad-mFasL; comprising portions of SEQ ID NO:4). In brief, mouse FasL cDNA, the sequence of which is represented herein by SEQ ID NO:11, was generated by RT-PCR (Bellgrau et al., 1995, *Nature* 377:630-632) and was inserted in the sense orientation into the pACCMV plasmid encoding the left end of the Ad5 chromosome (Gomez-Foix, et al., 1992, *J. Biol. Chem.* 267:25129-25134), but in which the CMV immediate early promoter replaces the E1 region.

The cell line 293, a human embryonic kidney cell line transformed by the E1 region of the adenovirus 5 chromosome, was purchased from the American Type Culture Collection (ATCC CRL-1573; Rockville, MD, USA) and was maintained in RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 7.5% heat-inactivated FBS (Hyclone, Logan UT, USA) and 2 mM L-glutamine (Gibco) at 37°C in 95% air and 5% CO_2.

Initial attempts to propagate the virus in the 293 cell line resulted in early death of the cells before significant viral titers could be obtained (data not shown). Greatly reduced viral titers were also reported by Muruve et al. in a similar attempt. The present inventors suspected that 293 cells may be undergoing apoptosis before significant viral replication occurred. To test this hypothesis, 293 cells were analyzed for Fas expression by flow cytometric immunofluorescence (Fig. 2A). Briefly, cell monolayers were harvested at 50-60% confluency using the trypsin-free chelating solution described below for ^{51}Cr-release experiments. The cells were washed once with PBS, pH 7.4, and once with the blocking solution (PBS with 5% goat serum and 0.1% sodium azide). 5 x 10^5 cells were placed in 1.5 ml microcentrifuge tubes, pelleted and resuspended in 200 μl of blocking solution containing either 2 μg/ml FITC-conjugated mouse IgG1 anti-human Fas receptor (clone DX2, Pharmingen, San Diego, CA, USA), or 2 μg/ml FITC-conjugated isotype control (clone DAK-G01, Dako Laboratories, Carpenteria, CA, USA). Samples were gently mixed and incubated at room temperature for 15 min, protected from light. Cells were washed once with PBS, fixed with 1% formaldehyde in PBS for 5 min, washed again with PBS and resuspended in 0.5 ml PBS for fluorescence analysis (University of Colorado Cancer Center

Flow Cytometry Core, UCHSC). The mean Fas fluorescence was 4.9-fold greater than nonspecific fluorescence. This value is relatively high compared to other cell lines that the present inventors have tested using the same method, and is similar to the level of Fas expressed by CEM cells.

Subsequent cytotoxicity experiments indicated that 293 cells are extraordinarily sensitive to the lytic effects of K562-hFasL (Fig. 2B), with complete lysis occurring at an E:T ratio of 10:1. Briefly, a standard chromium release assay was modified as follows. PC cell lines were removed from culture flasks by incubating at 37°C in a trypsin-free chelating solution (135 mM NaCl, 5 mM KCl, 20 mM HEPES, and 1.5 mM EDTA, pH 7.4). For each target cell line, 10^6 cells were pelleted and resuspended in 1 ml RPMI 1640 supplemented with 7.5% FBS and 10 mM HEPES buffer. Cells were labeled with 100 μCi ^{51}Cr as sodium chromate (ICN Pharmaceuticals Inc., Irvine, CA, USA) in this medium for 1 h at 37°C. Unincorporated ^{51}Cr was removed by washing twice in 10 ml medium, incubating for 1 h in fresh medium at 37°C, and washing again. Five thousand radiolabeled target cells in 100 μl were placed in each well of a round-bottomed 96-well tissue culture plate. Effector cells (K562-hFasL or K562-neo) were added at 10:1, 1:1, or 0.1:1 ratios to the target cells, also in a volume of 100 μl per well. The plates were incubated for 16-20 h at 37°C after which the cells were pelleted by centrifugation and 100 μl of cell-free supernatant were transferred to separate tubes for quantification of radioactivity using a gamma counter. Percent specific lysis was calculated using the following formula: (e-s/m-s) x 100, where e, s and m equal the amount of radioactivity released from PC cells incubated with effector cells (experimental lysis), with 100 μl 1% Triton X-100 (maximum lysis), respectively. Results in Fig. 2B are presented as the mean ± S.D. for triplicate samples.

Together, these data indicate that 293 cells were undergoing FasL-mediated apoptosis during the present inventors' attempts to produce Ad-mFasL virus. To overcome this problem, 293 cells were stably transfected with a plasmid encoding the cowpox virus caspase inhibitor crmA (SEQ ID NO:6) which inhibits FasL and TNF-mediated apoptosis. A modified calcium phosphate precipitation technique was used to transfect 293 cells with a pcDNA3-*crm*A construct generously provided by Dr. Tewari (University of Michigan, Ann

Arbor, Michigan). Stable transfectants, resistant to agonistic anti-Fas antibody-mediated apoptosis, were selected with Geneticin (Gibco) and pooled.

Recombinant virus was generated by transfecting 293-crmA cells with a mixture of pACCMV-mFasL and Ad5*dl*327 $_{Bst}\beta$-gal DNA-Terminal protein complex prepared from purified virions and digested with Bst-B1 which cleaves uniquely 3' of the LacZ coding sequence. Briefly, Ad5*dl*327$^{\beta st}\beta$-gal-TP complex was prepared by banding purified Ad5*dl*327$_{\beta st}\beta$-gal virions in 4 M guanidine-HCl (Sigma Chemical Company, St. Louis, MO), 2.8 M cesium chloride (Baxter, McGraw Park, IL). The gradient was fractionated and fractions containing Ad5*dl*327$_{\beta st}\beta$-gal-TP complex were collected and dialyzed against H_2O. The DNA-TP complex was then digested with BstBI (New England Biolabs, Beverly, MA), which cleaves uniquely 3' of the LacZ coding sequence (Schaack et al., 1995, *J. Virol* 6:3920-3923). The pACCMV-mFasL DNA was then mixed with BstBI-digested Ad5*dl*327$^{Bst}\beta$-gal-TP complex and was used to transfect 293-crmA cells using $Ca_3(PO_4)_2$ precipitation (Jordan et al., 1996, *Nucl. Acids Res.* 24:596-601). After 5 hr, the precipitate was removed and fresh medium was added. The transfected cells were incubated until the development of a strong cytopathic effect and freeze-thawed to release virus.

Dilutions of the cell lysate were used to infect 293-crmA cells, which were then overlaid with medium containing Noble agar (Difco, Detroit, MI, USA). After plaques developed, the cells were stained with neutral red (Baxter) and X-gal (Beohringer Mannheim, Indianapolis, IN, USA). Plaques that were clear in the presence of X-gal, and thus likely to be recombinants, were picked and grown in 293-crmA cells. Lysates of the plaque-infected cells were used to infect 293-crmA cells. Viral DNAs were isolated and restriction analysis used to select recombinant viruses encoding mFasL. Recombinant adenovirus encoding human FasL (the nucleic acid sequence encoding human Fas ligand is represented by SEQ ID NO:7)(the adenoviral vector construct is Ad-hFasL: SEQ ID NO:4) was generated using a similar strategy.

As shown in Fig. 2C, the resulting 293-crmA transfectants were almost entirely resistant to K562-hFasL allowing to effectively propagate adenoviruses encoding mouse and human FasL, yielding viral titers of at least 5 x 10^8 pfu/ml.

Example 11

The following example demonstrates the efficiency of adenovirus gene transduction in prostate cancer (PC) cells.

To be sure that adenovirus-mediated gene expression could be achieved efficiently in the PC cells, each cell line was transduced with 100 p.f.u./cell Ad-EGFP for 1 h. Ad5*dl*327CMV-EGFP (Ad-EGFP) encodes a humanized, enhanced, red-shifted jellyfish green fluorescent protein (Clontech Laboratories, Palo Alto, CA, USA) under the control of the CMV major immediate early promoter (J. Schaack et al., submitted). After 24 h of further incubation, the cells were analyzed for fluorescence by flow cytometry (Table 2). The transduction efficiency was high in six of the seven cell lines, with greater than 90% of the cells expressing EGFP. LNCaP was the only cell line that showed relatively poor adenoviral gene transduction, as measured by both the per cent positive cells (61%), and the relatively low fluorescence intensity obtained (13-fold brighter than control cells). Similar transduction efficiencies were obtained with each of the cell lines using a multiplicity of infection of 10 p.f.u./cell (data now shown). These data indicated that adenoviral transduction would be feasible in at least six of the seven PC cell lines.

TABLE 2

Efficiency of Ad-EGFP expression in seven PC cell lines. EGFP fluorescence was measured both by the per cent positive cells and the fold increase in mean fluorescent intensity as compared to untreated cells.

Cell line	% Positive cells	Fold increase in mean fluorescence
ALVA-31	99.6	118
JCA-1	98.7	172
PPC-1	98.1	235
DU 145	97.2	813
TSU-Pr1	93.2	55
PC-3	92.2	129
LNCaP	61.2	13

Example 12

The following example shows the effects of Ad-mFasL transduction on PC cell growth.

To determine how the PC cell lines would respond to internally expressed FasL, the growth of cell monolayers was measured for 1 week after transduction with either Ad-mFasL or Ad-EGFP as a control. In this experiment, cell monolayers were trypsinized and washed once with standard growth medium. For each cell line, 3.2 x 10^5 cells (control or 2.4 x 10^5 cells (Ad-EGFP or Ad-mFasL transduced) were placed into each of three conical Eppendorf tubes. Cells were pelleted by centrifugation, and supernatants were removed by aspiration. The pellets were resuspended in 500 µl of either plain medium (negative control), or 500 µl of medium containing approximately 10 or 100 p.f.u./cell of Ad-mFasL or Ad-EGFP. Cells were incubated for 1 h in a 37°C water bath with periodic mixing and were then washed twice with medium. The final cell pellets were resuspended in 16 ml medium (control cells) or 12 ml medium (Ad-mFasL and Ad-EGFP transduced cells). Each sample was then aliquotted into quadruplicate wells (1 ml each) of three 24-well tissue culture plates. To quantify the amount of DNA in the initial number of cells plated (i.e. Day 0), 1 ml aliquots of the negative control were placed in four Eppendorf tubes. Cells were pelleted, supernatant was aspirated, and the cell pellets were lysed in 0.25 ml 0.5 M NaOH. Cell lysates were then frozen at -20°C until all time points were collected. The remaining cultures were incubated at 37°C. Tissue culture medium was replaced every 48 h, and cells were harvested at the designated time points by aspirating the medium and lysing the monolayers in 0.5 M NaOH (0.25 ml/well), and freezing at -20°C. The DNA contents of the monolayers were quantified by Hoechst 33258 fluorescence using a Dynex Flurolite 1000 fluorescence plate reader (Dynex Technologies, Inc., Chantilly, VA, USA). DNA concentrations were calculated as the mean ± S.D. for triplicate determinations.

As shown in Fig. 3, ALVA-31 cells that were treated with Ad-EGFP show logarithmic growth as they approach confluency. A phase contrast photomicrograph was taken at day 4 post-infection (data not shown). Ad-EGFP treatment did not increase apoptosis above basal levels as confirmed by the lack of nuclear fragmentation apparent after staining cells with propidium iodide and Hoechst 33342 and observing under a fluorescent microscope (data not shown). In contrast, ALVA-31 cells that were treated with Ad-mFasL were nearly completely apoptotic within 24-48 h (Fig. 3) and the few remaining cells failed

to resume exponential growth over the course of this assay. Similar results were obtained with recombinant adenovirus encoding human FasL (data not shown). These data support the cytotoxicity results, suggesting that ALVA-31 cells are far more sensitive to the natural FasL protein than they are to agonistic anti-Fas antibody. Furthermore, this cell line appears to be more sensitive to internally expressed FasL than to that presented by K562-hFasL.

The short-term growth curves of other PC cell lines after adenoviral transduction are shown in Figs. 4A-4D. The cell line PPC-1 (Fig. 4A) behaves similarly to ALVA-31 in that FasL transduction nearly obliterates the entire population of cells, and positive growth is not detected over the course of the assay. FasL transduction was to varying extent less effective on JCA-1 (Fig. 4B), PC-3 (Fig. 4C) and TSU-Pr1 (Fig. 4D) cell growth in this *in vitro* assay. Interestingly, TSU-Pr1 is far more sensitive to FasL when it is presented by the K562-hFasL transfectant than when it is internally expressed. The reasons for this difference are not clear. However, two explanations are possible. First, TSU-Pr1, like certain other prostate cancers, may produce high amounts of matrix metalloproteinases resulting in the production of soluble FasL which is not functional. Second, TSU-Pr1 may not efficiently trimerize FasL into its functional form. The PC cell lines DU 145 and LNCaP yielded inconsistent results in the short-term growth assays with repeated trials (data not shown). For these reasons, although it is difficult to draw any conclusions regarding the sensitivities of these two cell lines to internally expressed FasL, recent results reported by Liu and colleagues suggest that these cell lines constitutively secrete soluble FasL which could interfere with FasL-mediated apoptosis following transduction (Liu et al., 1998, *Clin. Cancer Res.* 4:1803-1811).

Example 13

The following example demonstrates the effects of Ad-FasL on prostate tumor growth *in vivo*.

Although the ALVA-31 cell line appeared to be quite sensitive to apoptotic induction by Ad-mFasL, it was not clear if the few remaining cells would actually be capable of regenerating over a longer period of time, and if the cells would behave similarly in an *in vivo* environment after FasL transduction. To address these issues, an experiment was initiated to compare the growth of ALVA-31 cells with and without Ad-mFasL after 6 weeks

of growth intradermally in *Nu/Nu* mice. In this experiment, ALVA-31 and TSU-Pr1 cells were incubated alone (controls) or with recombinant adenoviruses (10 p.f.u./cell) for 60 min as described above for the growth assays. The cells were washed twice with tissue culture medium and once with PBS. Cell pellets were resuspended in PBS to yield a concentration of 3 x 10^6 cells per 100 µl (TSU-Pr1) or 2 x 10^6 cells per 100 µl (ALVA-31). For each PC cell line, four male *Nu/Nu* mice (National Cancer Institute, Bethesda, MD, USA), 6-8 weeks old, were injected intradermally with 100 µl of the cell suspensions in a total of five sites per mouse including both shoulders, both hips, and the center of the back. Each mouse, therefore, harbored three control tumors, one transduced with Ad-mFasL, and one transduced with Ad-EGFP.

When control tumors were approximately 0.5 cm in size (approximately 10 days later), two control tumors per mouse were injected in the centers with 50 µl free Ad-mFasL or Ad-EGFP virus (5 x 10^8 pfu/ml) to determine if regression or rejection could be initiated in an already established tumor. One mouse was then sacrificed 24 h later to histologically examine the tumors. Tumor sizes were measured using calipers. Two to six weeks after the start of the experiment, the remaining mice were sacrificed and the excised tumors were fixed in formalin and embedded in paraffin. Tissue sections were then stained with hematoxylin and eosin (Histology Laboratory, Department of Surgical Pathology, UCHSC). Histologic analyses were completed with the assistance of two objective and trained Pathologists, Drs. John Ryder and Rosina DeCampo (Department of Pathology, UCHSC).

Untreated ALVA-31 cells produced tumors in 9 of 12 injection sites. However, ALVA-31 cells that were infected with Ad-mFasL (10 p.f.u./cell) prior to injection failed to produce tumors in any of four sites. Also of interest in this preliminary set of experiments was the observation that the established control tumors could not be eradicated entirely by later injection of Ad-mFasL virus (10^7 p.f.u./tumor). Although localized apoptosis was apparent in tissue sections near the injection sites (data not shown), the virus may not have been sufficiently dispersed within the tumor to cause significant regression. This preliminary experiment raised several other important questions. For example, one question was whether the lack of tumor growth was due specifically to FasL expression, or whether the same effect

would be observed with Ad-EGFP. A second question was what were the *in vivo* effects of Ad-FasL in a prostate tumor cell line that appeared resistant *in vitro*.

To address these issues, a second set of experiments was carried out in mice using the PC cell line TSU-Pr1, whose growth was not inhibited by either Ad-EGFP or Ad-FasL *in vitro*. TSU-Pr1 cells were pre-infected with Ad-EGFP or Ad-mFasL as described above for the ALVA-31 cell line, and cells were injected intradermally into *Nu/Nu* mice. After 9 days, when control tumors were established, four control tumors were injected with Ad-EGFP or Ad-mFasL virus. One animal was then sacrificed 24 h later for histologic analysis. Calipers were used to measure the length and width of each tumor at days 9 and 18. The remaining animals were sacrificed after a total of 18 days. As shown in Table 3, there was no significant difference between the mean size of control and Ad-EGFP-infected TSU-Pr1 tumors either at day 9 or 18. In contrast, Ad-FasL tumors were significantly smaller and no change in tumor size was detected between days 9 and 18. Histologic analyses of these tumors revealed several surprising findings. First, Ad-FasL-treated tumors, although small, appeared viable, ruling out the possibility that scar tissue had completely replaced the tumor cells. Second, both Ad-FasL- and Ad-EGFP-treated tumors had extensive neutrophil infiltration (data not shown). This suggests that the initial infiltration of neutrophils is induced nonspecifically by adenoviral infection of tumor or other cells in the dermis. Furthermore, this infiltration is not in itself responsible for the regression/rejection of tumors in Ad-FasL-treated mice, as the tumors injected with Ad-EGFP were as large and as viable as control tumors. It was observed, however, that the neutrophils in the Ad-FasL-treated tumors often appeared apoptotic (data not shown). Thus, it is plausible that once the neutrophils are recruited in response to adenoviral infection, they undergo apoptosis in response to FasL, produced perhaps by dermal cells, and may potentiate a greater inflammatory response that indirectly suppresses TSU-Pr1 growth. Further experimentation will better define the role of neutrophils in mediating the rejection/regression of tumor cells that are not intrinsically sensitive to Ad-FasL mediated apoptosis.

TABLE 3

The effects of Ad-FasL on growth of TSU-Pr1 cells in nude mice. Cells were untreated (control), or pre-infected with Ad-EGFP or Ad-FasL. For comparison, several established control tumors were injected at day 10 with free Ad-EGFP or Ad-FasL virus.

TSU-Pr1 pretreatment	Day 9 Mean tumor area ± S.E.	Day 18 Mean tumor area ± S.E.
Control	9.7 ± 1.9	21 ± 10.3
Ad-EGFP	12.6 ± 2.3	27.5 ± 4.3
Ad-mFasL	2.3 ± 0.9	2.3 ±0.9
Injected Ad-EGFP	--	31 ± 3.9
Injected Ad-mFasL	--	23 ± 9.2

The data presented in the examples above indicate that several human PC cell lines are significantly more sensitive to FasL-mediated apoptosis than was originally reported with the use of agonistic anti-Fas antibodies. Furthermore, the majority of these cell lines respond best to FasL when it is expressed internally via the adenoviral system. This method of internal expression may better represent what occurs in the prostate *in vivo* since the prostatic epithelium has been shown to coexpress Fas and FasL. The present inventors' *in vivo* experiments suggest therapeutic potential for FasL transgene expression in treating cancer patients. Given the prevalence of prostate cancer and the limited effectiveness of available therapies, further research in this area seems warranted.

While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention, as set forth in the following claims.